NIH061.1CP1C2 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Lori et al.	Group Art Unit 1623
Appl. No.	:	09/756,411	
Filed	:	January 8, 2001	
For	:	NEW PROCEDURE TO BLOCK THE REPLICATION OF REVERSE TRANSCRIPTASE DEPENDENT VIRUSES BY THE USE OF INHIBITORS OF DEOXYNUCLEOTIDES SYNTHESIS	
Examiner	:	Crane, L.E.	

DECLARATION UNDER 37 CFR 1.132 OF DR. FRANCO LORI

I, Dr. Franco Lori, do hereby declare:

1. I am a named inventor of the above-identified application. A true and correct copy of my Curriculum Vitae is attached as Exhibit 1.

2. We can show 2 HU analogues, both inhibitors of ribonucleotide reductase, that behave like HU: trimidox (Sumpter *et al.* 2004 *Antiviral Research* **62**:111-112) and didox (Sumpter *et al.* 2004 *Antiviral Research* **62**:111-112).

3. We can show 2 antiviral nucleoside phosphate analogues (other than a thymidine or cytidine analogue) that behave like ddI: abacavir, a guanosine analogue (Sumpter *et al.* 2004 *Antiviral Research* **62**:111-112) and PMEA, a adenosine analogue (Palmer *et al.*, 1999 *Antimicrobial Agents and Chemotherapy* **43**:2046-2050).

4. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or patent issuing therefrom.

Respectfully submitted,

Dated: 08/17/04

By: _____
Dr. Franco Lori

O:\DOCS\EBI\SB1-1517.DOC
081004

CURRICULUM VITAE
FRANCO LORI, M.D., Ph.D.

Highlights

- over 20 years of research experience in the field of molecular biology, virology, immunology and clinical research, including
    - Antiretroviral therapy
    - Immunomodulators
    - Gene therapy of AIDS
    - Genetic Immunization
- 14 years management experience in the biotech industry
    - 4 years Vice-President of Genetic Immunity, LLC
    - 10 years Board of Director & management of RIGHT

- 13 years experience in HIV drug development
- 12 years experience in supervising multidisciplinary teams to rapidly and cost effectively proof the principle of new drugs and treatment approaches
- Exceptional ability in synthesizing new research findings and applying the knowledge to drug development
- Experience in investigative pre-clinical and clinical research
- Experience in regulatory affairs of drugs and biologicals development
- Widespread network with clinical-, pharmaceutical-, government- and basic researchers in US and Europe

Professional Experience

Scientific Co-Director and Founder, RIGHT (1994-Present)
Research Institute for Genetic and Human Therapy (RIGHT), Washington, DC and Pavia, Italy

- Managing the company business in the USA
- Strategic planning and supervising of the basic and clinical HIV therapy research
- Designing, coordinating and co-chairing Phase I/II clinical trials
- Negotiating the contract and managing the CRO during the clinical trials
- Investigating primary HIV infection including the Berlin Patient
- Designing primate experiments for structured treatment interruptions (STI) and supervising scientists throughout the projects
- Managing the in vitro and primate development of DermaVir therapy
- Supervising immunological assay development for HIV-specific T cell responses
- Supervising the immunological evaluation of human and primate trials
- Co-chairing clinical trials with HU and STI in Italy
- Coordinating the research projects with collaborators
- Writing and co-author of manuscripts on prevalent findings
- Presenting results in international meetings

- Writing patent applications and supervising IP counsel
- Overseeing the administrative operations
- Strategic planning for the allocation of funds
- Fundraising, including writing, presentations, submitting grant proposals, and coordinating public relations

Vice-President and Founder, Genetic Immunity, LLC (1998-Present)
Washington, DC (www.geneticimmunity.com)

- Fundraising
- Presenting the company to investors
- Supervising the production of company presentation material
- Public relations
- Strategic planning and business development
- Managing the company business
- Designing the Product Development Plan for DermaVir
- Overseeing the pre-clinical toxicity study of DermaVir
- Participating in the adult and pediatric Phase I/II DermaVir trials with ACTG and PACTG
- Managing the pre-IND and IND submissions
- Coordinating the research projects with collaborators

Adjunct Associate Professor of Microbiology & Immunology (1996 - present)
Georgetown University, Washington, D.C.

1st Level Director (1995 - present)
IRCCS Policlinico S. Matteo, Pavia, Italy

- Lecturing students and faculty on AIDS therapy
- Organizing lectures on chemotherapy and gene therapy for educational purposes
- Supervising Italian facilities of RIGHT

Visiting Associate (1994)
Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

- Pharmacological new approaches to AIDS
- Molecular Pharmacology
- Gene Therapy of AIDS

Adjunct Professor at the School of Medicine (1993)
University of Verona, Italy

- Teaching course on genetic approaches to AIDS therapy

Visiting Fellow, Laboratory of Tumor Cell Biology (1990 - 1993)
National Cancer Institute, National Institutes of Health, Bethesda, Maryland (USA)

- Analysis of the early phases of HIV-1 replication

- Relationship of HIV-1 integration status to gene expression
- Study of genetic determinants of HIV-1 cell tropism

Guest Researcher, Laboratory of Tumor Cell Biology (1989-1990)
National Cancer Institute, National Institutes of Health, Bethesda, Maryland (USA)

- Study of the biochemistry and molecular biology of transcription

Post-Doctoral Fellow, Institute of Genetics of Council of National Research and Institute of Infectious Diseases (1987-1988)

- Analysis of polymerases in human leukemia
- Gel analysis of HIV-1 proteins

Clinical Resident, Institute of Medical Pathology (1983-1986)
University of Parma, Italy

- Routine care of patients in Internal Medicine

Education

Ph.D. (Hematology), July 1986, magna cum laude
University of Pavia, Italy
Thesis: Polymerases α and β in Human Leukemia

M.D., June, 1983, magna cum laude
University of Parma, Italy

Volunteer Assistant (1983-1986)
Institute of Medical Pathology, University of Parma, Italy

Intern in Medicine (1979-1983)
University of Parma, Italy

Honors and Other Special Recognitions

2003	Member of the International Scientific Committee of the 1st International Workshop on HIV Persistence During Therapy, Saint Martin, French West Indies
2003	Member of the International Scientific Committee of the International Conference "Immune Reconstitution and Control of HIV", Stresa, Italy
2002	Member of the Review Board for the XIV International AIDS Conference, Barcelona, Spain
2001	Member of the Scientific Advisory Board of the Global Healthcare Organization
2000	Heroes in Medicine Award from the International Association of Physicians in AIDS Care

1996	Member of the International Scientific Committee of XI International Conference on AIDS
1993	Technology Transfer Award from National Institutes of Health
1990-93	Ministry of Health of Italy, Award for AIDS Research
1989	International Travel Award, USA-Italy Cooperative Treaty Technology Transfer Award from National Cancer Institute
1986	Paola Dalla Rosa Fellowship, University of Parma and European, School of Oncology
1985	Oreste Battioni Fellowship, Italian Cancer Association
1984	Roberto Burlenghi Degree Award, University of Parma

Recent Invited Presentations to International Conferences

- IV World Congress on Vaccines and Immunisation, Tokyo, Japan (2004)
- XV International AIDS Conference, Bangkok, Thailand. (2004)
- 1st International Workshop on HIV Persistence during Therapy, Saint Martin, French West Indies (2003)
- IV International Workshop on HIV Eradication, Badalona, Spain. (2003)
- Spanish Virology Research Meeting, Sevilla, Spain. (2003)
- Distinguished Faculty in HIV Conference, New York, USA. (2003)
- HIV DART 2002, Frontiers in Drug Development for Antiretroviral Therapies, Naples, Florida. (2002)
- 2002 FASEB Summer Research Conferences "Therapeutic and Preventive AIDS Vaccines", Tucson, Arizona. (2002)
- IAPAC Sessions 2002. University of Chicago Gleacer Center, Chicago (2002)
- XIV International AIDS Conference, Barcelona (2002)

Inventor; Selected Patents and Patent Applications

1. Published application: PCT/US91/00175, filed 16 Jan 91, "Vector with Multiple Target Response Elements Affecting Gene Expression."

2. Published application: PCT/US92/04011, filed 21 May 92, "Eukaryotic Expression Vectors with Regulation of RNA Processing."

3. Published application: PCT/US95/05955, filed 18 May 95, "Vectors with Multiple HIV-1 TAR Sequences Inhibiting HIV-1 Gene Expression."

4. Published application: PCT/US97/02933, filed 20 Feb 97, "Methods and Compositions for Protective and Therapeutic Genetic Immunization."

5. Published application: PCT/US98/04219, filed 4 May 98, "Chimeric RNA having Synergistic Anti-HIV Activity."

6. Published application: PCT/US99/03454, filed 18 Feb 99, "Method of Rendering A Human Immunodeficiency Virus Population Replication Incompetent *in vivo*."

7. Published application: PCT/US99/03452, filed 18 Feb 99, "Compositions for Rendering a Human Immunodeficiency Virus using Hydroxyurea and a Reverse Transcriptase Inhibitor *in vivo*."

8. US Patent No. 5,977,086 issued 2 Nov 99, "Method of Inhibiting Human Immunodeficiency Virus by Combined Use of Hydroxyurea, a Nucleoside Analog, and a Protease Inhibitor."

9. Published application: PCT/US00/02754, filed 2 Feb 2000, "Use of Hydroxyurea and a Reverse Transcriptase Inhibitor to Induce Autovaccination by Autologous HIV"

10. US Patent No. 6,114,312 issued 5 Sep 2000, "Method of Inhibiting Human Immunodeficiency Virus by Combined Use of Hydroxyurea, a Nucleoside Analog, and a Protease Inhibitor."

11. US Patent No. 6,130,089 issued 10 Oct 2000, "Materials and Methods for Gene Transfer."

12. Patent No. Pending, "Vector with Multiple Target Response Elements Affecting Gene Expression."

13. US Patent No. 6,420,176 B1 issued 16, July 2002, "Composition for Delivering DNA into Antigen Presenting Cells."

Other Experiences

- Italy: co-director of Italian RIGHT since 1995; 1997-2001 leader of a European Gene Therapy Network (Italy, Nederland, France, Belgium)
- USA: Franco Lori speaks fluent English, has been living in Washington DC for 14 years. Worked in the National Cancer Institute in Dr. Gallo's laboratory (co-discoverer of HIV).

Publications

1. Bonati, A., **Lori F.**., Buscaglia, M., and Starcich, R.: The Phenotype of Terminal Deoxynucleotidyl Transferase Positive Cells in the Hemopoietic Tissues of Human Fetuses. In Torelli, U., Di Prisco, A.U., and Castaldini, C. (Eds.): Frontiers in Experimental Hematology. Rome, Serono Symposia Publications, 1983, pp. 210-219.

2. **Lori F.**., Casoli, C., Albertini, R., Bonati, A., and Starcich, R.: Analysis of Catalytic Subunits of DNA Polymerase α in Acute Myeloblastic Leukemia. In Cajozzo, A. and Perricone, R. (Eds.): Attualita' in Ematologia. Bologna, Monduzzi, 1985, pp. 120-129.
3. **Lori F.**., Casoli, C., and Starcich, R.: DNA Polymerase α Activity in Acute Leukemia: Comparative Analysis of Catalytic Subunits after SDS-PAGE. Boll. Soc. It. Biol. Sper. 62(10):1197-1203, 1986.
4. Casoli, C., **Lori F.**., and Starcich, R.: Heterogeneity of DNA Polymerase α in Embryo Tissues. Boll. Soc. It.al Biol. Sper. 62(10):1205-1211, 1986.
5. **Lori F.**., Scovassi, A.I., Brusamolino, E., Casoli, C., and Starcich, R.: Different Expression of DNA Polymerase α and β in Bone Marrow and Peripheral Blood in Acute Leukemia. In Habmont, A.C. (Ed.): Attualita' in Ematologia, 1987. Bologna, Monduzzi, 1987, pp. 747-750.
6. Casoli, C., Tremolada, F., **Lori F.**., Scovassi, I., Bertazzoni, U., Starcich, R., Realdi, G., and Alberti, A.: Detection and Characterization of Reverse Transcriptase Activity in Sera of Patients with Post-transfusion Non-A Non-B Hepatitis. Ital. J. Gastroenter. 19: 31, 1987.
7. Casoli, C., Tremolada, F., **Lori F.**., Scovassi, I., Bertazzoni, U., Starcich, R., and Alberti, A.: Reverse Transcriptase Activity in Post-transfusion Non-A Non-B Hepatitis. I° Characterization and Association with Retrovirus-like Particles. Serodiagnosis and Immunotherapy 1: 339-346, 1987.
8. **Lori F.**., Scovassi, A.I., Brusamolino, E., Casoli, C., and Starcich, R.: Different Distribution of DNA Polymerases α and β in Bone Marrow and Peripheral Blood from Human Acute Leukemia. Med. Oncol. Tumor Pharmacother. 5(3): 181-186, 1988.
9. **Lori F.**., Scovassi, A.I., Zella, D., Achilli, G., Cattaneo, E., Casoli, C., and Bertazzoni, U.: Enzymatically Active Forms of Reverse Transcriptase of the Human Immunodeficiency Virus. AIDS Res. Hum. Retroviruses 4(5): 393-398, 1988.
10. Bertazzoni, U., **Lori F.**., Achilli, G., and Cattaneo, E.: Assays and Structure of Reverse Transcriptase of the AIDS Virus. Clin. Chim. Acta 183(1): 101-106, 1989.
11. **Lori F.**., Achilli, G., Cattaneo, E., Casoli, C., and Bertazzoni, U.: Usefulness of Activity Gel Analysis in the Study of Reverse Transcriptase of HIV-1 and other Retroviruses. Arch. AIDS Res. 4: 1-8, 1990.
12. Zella, D., Mori, L., Sala, M., Ferrante, P., Casoli, C., Magnani, G., Achilli, G., Cattaneo, E., **Lori F.**., and Bertazzoni, U.: HTLV-II Infection in Italian Drug Abusers. Lancet 366(8714): 575-576, 1990.
13. Lusso, P., **Lori F.**, and Gallo, R.C.: CD-4 Independent Infection by Human Immunodeficiency Virus Type-1 after Phenotyping Mixing with Human T-cell Leukemia Viruses. J. Virol. 64(12): 6341-6344, 1990.
14. Lusso, P., De Maria, A., Malnati, M., **Lori F.**., DeRocco, S.E., Baseler, M., and Gallo, R.C.: Induction of CD4 and HIV-1 Susceptibility in Human CD8 + T Lymphocytes by HHV-6. Nature 349(6309): 533-535, 1991.
15. **Lori F.**., Hall, L., Lusso, P., Popovic, M., Markham, P., Franchini, G., and Reitz, M.S., Jr. : Effect of Reciprocal Complementation of two Defective Human Immunodeficiency Virus Type- 1 (HIV-1) Molecular Clones on HIV-1 Cell Tropism and Virulence. J. Virol. 66(9): 5553-5560, 1992.
16. **Lori F.**., di Marzo Veronese, F., De Vico, A.L., Lusso, P., Reitz, M.S., and Gallo, R.C.: Viral DNA Carried by HIV-1 Virions. J. Virol. 66(8): 5067-5074, 1992.
17. Zella, D., Cavicchini, A., Salemi, M., Casoli, C., **Lori F.**., Achilli, G., Cattaneo, E., Landini, V., and Bertazzoni, U.: Molecular Characterization of two Isolates of Human T-cell Leukemia Virus Type-II from Italian Drug Abusers and Comparison of Genome Structure with other Isolates. J. Gen. Virol. 74: 315-319, 1993.
18. Lisziewicz, J., Sun, D., Smythe, J., Lusso, P., **Lori F.**., Louie, A., Markham, P., Rossi, J., Reitz, M., and Gallo, R.C.: Inhibition of Human Immunodeficiency Virus Type I Replication by Regulated

Expression of a Polymeric-TAR RNA Decoy as a Strategy for Gene Therapy. Proc. Natl. Acad. Sci. USA 90(17): 8000-8004, 1993.

19. Gao, W-Y., Cara, A ., Gallo, R.C., and **Lori F.**.: Low Levels of Deoxynucleotides in Peripheral Blood Lymphocytes: A Strategy to Inhibit Human Immunodeficiency Virus Type-1 Replication. Proc. Natl. Acad. Sci. USA 90(19): 8925-8928, 1993.
20. **Lori F.**., Lisziewicz, J., Smythe, J., Cara, A., Bunnag, T.A., Curiel, D., and Gallo, R.C.: Rapid Protection Against Human Ummunodeficiency Virus Type 1 (HIV-1) Replication Mediated by High Efficiency Non-retroviral Delivery of Genes Interfering with HIV-1 tat and gag. Gene Therapy 1(1): 27-31, 1994
21. **Lori F.**. , Malykh, A., Cara, A., Sun, D., Weinstein, J.N., Lisziewicz, J., and Gallo, R.C.: Hydroxyurea as an Inhibitor of Human Immunodeficiency Virus-type 1 Replication. Science 266(5186): 801-805, 1994.
22. Malykh, A., Louie, A., Hall, L., Reitz, M. and **Lori F.**. Multiple Hierarchic Determinants for Growth of HIV-1 in Monocyte-macrophage. Virology 206(1): 646-650, 1995.
23. Cara, A., Guarnaccia, F., Reitz, M.S. , Gallo, R.C., and **Lori F.**.: Self-limiting, Cell-type Dependent Replication of an Integrase Defective HIV-1 in Human Macrophages but not in T-lymphocytes. Virology 208: 241-248, 1995.
24. **Lori F.**. and Gallo, R.C.: Hydroxyurea and AIDS: An Old Drug Finds a New Application? AIDS Res. and Hum. Retroviruses 11(10): 1149-1151, 1995.
25. Villani, P., Maserati, R., Regazzi-Bonora, M., Giacchino, R., and **Lori F.**.: Pharmacokinetics of Hydroxyurea in Patients Infected with Human Immunodeficiency Virus Type I. J. Clin. Pharmacol. 36(2): 117-121, 1996.
26. Cara, A., Cereseto, A., **Lori F.**., Reitz, M.: HIV-1 Protein Expression from Synthetic Circles of DNA Mimicking the Extrachromosomal Forms of Viral DNA. The Journal of Biological Chemistry 271(10): 5393-5397, 1996.
27. Lisziewicz, J., Sun, D., Gallo, R., Ensoli, B. and **Lori F.**.: Efficacy of *antitat* Gene Therapy in the Presence of High Multiplicity Infection and Inflammatory Cytokines. Human Gene Therapy, 7(18):2209-2216, 1996.
28. Foli A., **Lori F.**., Maserati, R., Tinelli, C., Minoli, L., Lisziewicz, J.: Hydroxyurea (HU) and Didanosine (ddI) as a More Potent Combination than HU and Zidovudine (AZT). An In Vitro Prediction in Confirmed in Vivo. Antiviral Therapy 2:31-38, 1997.
29. **Lori F.**., Jessen, H., Foli, A., and Lisziewicz J.: Long Term Suppression of HIV-1 by Hydroxyurea and Didanosine. JAMA 277(18):1437-38, 1997.
30. De Antoni, A., Foli, A., Lisziewicz, J., **Lori F.**.: Mutations in the pol Gene of Human Immunodeficiency Virus Type 1 in Infected Patients Receiving Didanosine and Hydroxyurea Combination Therapy. J of Infectious Diseases 176:899-903, 1997
31. **Lori F.**., Gallo, R.C., Malykh, A., Cara, A., Romano, J., Markham, P.: Didanosine but not High Doses of Hydroxyurea Rescue Pigtailed Macaque from a Lethal Dose of $SIV_{smmpbj14}$. AIDS Research and Human Retroviruses, 13 (13):1083-88, 1997
32. **Lori F.**., Malykh, A., Foli, A., Maserati, R., De Antoni, A., Minoli, L., Padrini, D., Degli Antoni, A., Baerchi, E., Jessen, H., Wainberg, M., Gallo, R., Lisziewicz, J.: Combination of a Drug Targeting the Cell with a Drug Targeting the Virus Controls Human Immunodeficiency Virus Type 1 Resistance. AIDS Research and Hum. Retroviruses 13 (16): 1403-1409, 1997
33. Foli, A., Maserati, R., Minoli, L., Wainberg, M.A., Gallo, R.C., Lisziewicz, J., **Lori F.**.: Therapeutic Advantage of Hydroxyurea and Didanosine Combination Therapy in Patients Treated with Zidovudine. AIDS, 1998.
34. Lisziewicz, J., Jessen, H., Finzi, D., Siliciano, R.F., **Lori F.**.: HIV-1 Suppression by Early Treatment with Hydroxyurea, Didanosine, and a Protease Inhibitor. Lancet 352 (9123): 199-200, 1998

35. **Lori F.**., Lisziewicz J.: Cellular Factors: Targets for the Treatment of HIV Infection. Antiviral Therapy 3: 79-90, 1998
36. **Lori F.**., Lisziewicz J.: Hydroxyurea: Mechanisms of HIV-1 Inhibition. Antiviral Therapy 3 (Supplement 4): 26-33, 1998
37. Boyer P.L., Lisziewicz, J., **Lori F.**., Hughes, S.H.: Analysis of Amino Insertion Mutations in the Fingers Subdomain of HIV-1 Reverse Transcriptase. Journal of Molecular Biology 286(4):995-1008, 1999.
38. Finzi, D., Blankson, J., Siliciano, J.D., Margolick, J.B., Chadwick, K., Pierson, T., Smith, K., Lisziewicz, J., Siliciano, R.F.: Latent Infection of CD4+ T Cells Provides a Mechanism for Lifelong Persistence of HIV-1, Even in Patients on Effective Combination Therapy. Nature Medicine (5)5: 512-517, 1999
39. Foli, A. and **Lori F.**.: Hydroxyurea Combination Therapy. Journal of HIV Therapy. 4(2): 45-48, 1999.
40. D'Aquila, R.T., Johnson, V.A., **Lori F.**., Palmer, S.: Drug Resistance and Pathogenesis: Clinical Implications of New Data. Massachusetts General Hospital, 1-17, 1999
41. **Lori F.**, Jessen H., Lieberman J., Clerici M., Tinelli C., Lisziewicz J: Immune Restoration by Combination of a Cytostatic Drug (Hydroxyurea) and Anti-HIV Drugs (ddI and Indinavir). AIDS Res Human Retroviruses, 15 (7), 619-624, 1999.
42. Lisziewicz, J., Rosenberg, E., Lieberman, J., Jessen, H., Lopalco, L., Siliciano, R., Walker, B. and **Lori F.**.: Control of HIV Despite the Discontinuation of Antiretroviral Therapy. New England J of Medicine. 340(21): 1683-4, 1999.
43. **Lori F.**: Hydroxyurea and HIV: 5 Years Later-- From Antiviral to Immune-modulating Effects. AIDS. 13(12): 1433-42, 1999.
44. Seminari E., Lisziewicz J., Tinelli C., Foli A., **Lori F.**., Maserati, R.: Hydroxyurea Toxicity when Combined with Didanosine (ddI)- in HIV-1 Seropositive Asymptomatic Individuals. Int'l J. of Clin Pharmaceutical and Therapeutics. 37(10): 514-518, 1999.
45. **Lori F.** Hydroxyurea for the Therapy of HIV Infection: From Antiviral to Immune-Modulating Effects. The PRN Notebook. 4(2): 9-12, June 4, 1999.
46. Ravot, E., Lisziewicz, J. and **Lori F.**: New Uses for Old Drugs in HIV Infection: The Role of Hydroxyurea, Cyclosporin and Thalidomide. Drugs. 58(6): 953-63, 1999.
47. Lisziewicz, J and **Lori F.**.: Mechanisms of Human Immunodeficiency Virus Type I Inhibition by Hydroxyurea. Journal of Biological Regulators and Homeostatic Agents, 1999. 13(3): 176-80, 1999 *Review.*
48. **Lori F.**., Jessen, H., Lieberman, J., Finzi, D., Rosenberg, E., Tinelli, C., Siliciano, R., Walker, B. and Lisziewicz, J.: Treatment of Human Immunodeficiency Virus Infection with Hydroxyurea, Didanosine, and a Protease Inhibitor before Seroconversion is Associated with Normalized Immune Parameters and Limited Viral Reservoir. Journal of Infectious Diseases. 180(6): 1827-1832, 1999.
49. **Lori F.**, and Lisziewicz J. Role of Immune Modulation in Primary HIV Infection. Journal of Biological Regulators and Homeostatic Agents. 25: 45-48, 2000.
50. **Lori F.**, Rosenberg E., Lieberman J., Foli, A. Maserati, R., Seminari, E., Alberici, F., Walker B., Lisziewicz J.: Hydroxyurea and Didanosine Long-Term Treatment Prevents Breakthrough and Normalizes Immune Parameters. AIDS Res. Hum Retr. 15(15):1333-1338, 1999
51. Lieberman, J., Trimble, L.A., Friedman, R.S., Lisziewicz, J., **Lori F.**., Shankar, P., Jessen, H.: Expansion of CD57 and CD62L-CD45RA+CD8 T Lymphocytes Correlates with Reduced Viral Plasma RNA after Primary HIV Infection. AIDS 13(8) 1999.
52. **Lori F.** and Lisciewicz J. Targeting HIV Reservoirs and Reconstituting the Immune System. AIDS Research and Human Retroviruses. 15(18):1597-1617 1999.

53. Boyer, P.L., Lisziewicz, J., **Lori F.**., Hughes, S.H.: Analysis of Amino Insertion Mutations in the Fingers Subdomain of HIV-1 Reverse Transcriptase. J. of Molecular Biology 286, 995-1008, 1999.
54. **Lori F.**. and Lisziewicz, J.: Hydroxyurea Overview of Clinical Data and Antiretroviral and Immunomodulatory Effects. Antiviral Therapy. 4(3): 101-108, 1999.
55. **Lori F.**., Maserati, R., Foli, A., Seminari, E., Timpone, J., Lisziewicz, J.: Structured Treatment Interruptions to Control HIV-1Infection, The Lancet, 355(9200): 287-288, 2000.
56. Lisziewicz, J., Zeng, G., Gratas, C., Weinstein, J.N., **Lori F.**.: Combination Gene Therapy: Synergystic Inhibition of Human Immunodeficiency Virus Tat and Rev Functions by a Single RNA Molecule. Human Gene Therapy. 11: 807-815, 2000.
57. Barreio, P. and **Lori F.**.: The Role of Hydroxyurea in the Treatment of HIV Infection. AIDS Reviews. (2) 99-104, 2000.
58. **Lori F.** and Lisziewicz J.: Rationale for the Use of Hydroxyurea as Anti-Human Immunodeficiency Virus Drug. Clinical Infectious Diseases, 30(S2): S193-S197, 2000.
59. Ravot E., Tambussi G., Jessen H., Tinelli C., Lazzarin A., Lisziewicz J., **Lori F.** Effects of Hydroxyurea on T Cell Count Changes During Primary HIV Infection. AIDS 14(5):619-622, 2000.
60. **Lori F.**., Lewis, M.G., Xu, J., Varga, G., Zinn, D.E., Crabbs, C., Wagner, W., Greenhouse, J., Silvera, P., Yalley-Ogunro, J., Lisziewicz, J.: Control of SIV Rebound Through Structured Treatment Interruptions During Early Infection. Science 290(5496) 1591-1593, 2000.
61. Foli, A., Benvenuto, F., Piccinini, G., Bareggi A., Cossarizza, A., Lisziewicz, J., and **Lori F.** Direct analysis of mitochondrial toxicity of antiretroviral drugs. AIDS. Sep. 2001 15(13), 1687-1694, 2001.
62. Lisziewic, J., Gabrilovich DI, Varga, G., Xu, J., Greenberg, PD., Arya, SK, Bosch, M., Behr, JP. and **Lori F.**. Induction of potent human immunodeficiency virus type 1-specific T-cell-restricted immunity by genetically modified dendritic cells. J. Virol. Aug. 2001 75(16):7621-8, 2001.
63. **Lori F.**. and Lisziewicz J. Structured Treatment Interruptions for the Management of HIV Infection in 2001. JAMA. Dec 19;286(23):2981-7, 2001.
64. Lisziewicz, J., Galluzzi, L and **Lori F.** Gene Therapy for HIV-1. Bundesgesundheitsbl 44:1071-1075, 2001.
65. **Lori F.** and Lisziewicz J. Structured Treatment Interruptions for HIV-AIDS therapy. International Antiviral News 2001 9(7): 118-120, 2001.
66. Piccinini G., Foli A., Comolli G., Lisziewicz J. and **Lori F.** Complementary Antiviral Efficacy of Hydroxyurea and Protease Inhibitors in HIV-Infected Dendritic Cells and Lymphocytes. J. Virol. Mar; 76(5):2274-8, 2002.
67. Xu J., Whitman L., **Lori F.** and Lisziewicz J. Methods of Using IL-2 to Enhance HIV-specific Immune Responses. AIDS Research and Human Retroviruses. Mar; 18(4):289-293, 2002.
68. Lisziewicz J. and **Lori F.** Structured Treatment Interruptions in HIV/AIDS Therapy. Journal of Microbes and Infection. Feb; 4(2):207-14, 2002.
69. Ravot, E., Comolli, G., **Lori F.**. and Lisziewicz J. High efficiency lentiviral gene delivery in non-dividing cells by deoxynucleoside treatment. Journal of Gene Medicine. Mar-Apr; 4(2):161-9, 2002.
70. **Lori F.**, Foli A., Maserati R., Seminari E., Xu J., Whitman L., Ravot E., Alberici F., Lopalco L., and Lisziewicz J. Control of HIV during a structured treatment interruption in chronically infected individuals with vigorous T cell responses. HIV Clinical Trials Mar-Apr;3(2):115-24, 2002.
71. Foli A., Seminari E., Ravot E., Lisziewicz J. and **Lori F.** Role of Hydroxyurea During Structured Treatment Interruptions. Journal of Biological Regulators and Homeostatic Agents Jan-Mar;16(1):64-8, 2002.
72. **Lori F.**, Foli A. and Lisziewicz J. Structured Treatment Interruptions as a Potential Alternative Therapeutic Regimen for HIV Infected Patients: a Review of Recent Clinical Data and Future Prospects. Journal of Antimicrobial Agents. 50:155-160, 2002.
73. Xu J., Whitman L., **Lori F.**, and Lisziewicz, J. Quantification of HIV-specific CD8+ T Cells by in vitro Simulation of Viral Rebound. AIDS. 16(14):1849-1858, 2002.

74. **Lori F.**, Guallini P., Galluzzi L. and Liszievicz J. Gene Therapy Approaches to HIV Infection. Pharmacogenomics. 2(4):245-52, 2002.
75. Lisziewicz J., Bakare, N., **Lori F.**.: Vaccination for the treatment of HIV/AIDS. Vaccine. Jan 30;21(7-8):620-3, 2003
76. Garcia F, Plana M, Arnedo M, Ortiz GM, Miro JM, Lopalco L, **Lori F.**, Pumarola T, Gallart T, Gatell JM. A cytostatic drug improves control of HIV-1 replication during structured treatment interruptions: a randomized study. AIDS. Jan 3;17(1):43-51, 2003
77. Xu, J. **Lori, F.**, Lisziewicz, J.: Vaccinia assay for the rapid detection of functional HIV-specific CD8+ cytotoxic T lymphocytes. J Immunol Methods. 276(1-2): 45-57, 2003.
78. Lisziewicz, J., Wainberg, M., and **Lori, F.**: Is There a Role for Hydroxyurea in the Treatment of HIV Infection? Drug Safety. 26(9):605-24, 2003.
79. Lisziewicz, J. and **Lori, F.**: Meeting Highlights: 2nd International Symposium on Molecular Diagnostics and Skin Gene Therapy. Expert Opinion on Biological Therapy (EOBT). Jul;3(4):683-7, 2003.
80. **Lori, F.**, Kelly, L.M. and Lisziewicz, J.: Immunological Approaches for HIV Gene Therapy. Curr Drug Targets Infect Disord. Jun;3(2):171-8, 2003.
81. Richardson MW, Sverstiuk AE, Silvera P, Greenhouse J, Lisziewicz J, **Lori F**, Khalili K, Lewis MG, Rappaport J.: T-cell receptor excision circles (TREC) in SHIV 89.6p and SIVmac251 models of HIV-1 infection. DNA Cell Biol. Jan;23(1):1-13, 2004.
82. **Lori, F.** Maserati, R., Foli, A., Barasolo, G., Castelli, F., Tomasoni, L., Migliorino, M., Maggiolo, F., Pan, A., Paolucci, S., Scudeller, L., Tinelli, C., D'Aquilla, R., Lisziewicz, J.: Strategies to decrease viral load rebound and prevent loss of CD4 and onset of resistance during structured treatment interruptions. Antiviral Therapy. Feb;9(1):123-32, 2004.

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Antiviral Research 62 (2004) 111–120
Antiviral Research
www.elsevier.com/locate/antiviral

In vivo examination of hydroxyurea and the novel ribonucleotide reductase inhibitors trimidox and didox in combination with the reverse transcriptase inhibitor abacavir: suppression of retrovirus-induced immunodeficiency disease

L. Ryan Sumpter[a], Mohammed S. Inayat[a], Erin E. Yost[a], William Duvall[a], Espen Hagan[a,b], Christopher N. Mayhew[a], Howard L. Elford[c], Vincent S. Gallicchio[a,*]

[a] *Department of Clinical Sciences, University of Kentucky Medical Center, CHS Building, 900 South Limestone Street, Lexington, KY 40536, USA*
[b] *Aalessund University, Aalessund, Norway*
[c] *Molecules for Health Inc., Richmond, VA, USA*

Received 14 July 2003; accepted 10 November 2003

Abstract

Inhibition of ribonucleotide reductase (RR) has gained attention as a potential strategy for HIV-1 therapy through the success of hydroxyurea (HU) to potentiate the activity of the nucleoside reverse transcriptase inhibitor (NRTI) didanosine (ddI) in clinical trials. However, the use of HU has been limited by its development of hematopoietic toxicity. In this study, the novel RR inhibitors didox (DX; 3,4-dihydroxybenzohydroxamic acid), and trimidox (TX; 3,4,5-trihydroxybenzamidoxime) were evaluated along with HU for anti-retroviral efficacy in LPBM5-induced retro-viral disease (MAIDS) both as monotherapeutic regimens and in combination with the guanine containing NRTI abacavir (ABC). Anti-retroviral drug efficacy was determined by measuring inhibition of splenomegaly, hypergammaglobulinemia, and splenic levels of proviral DNA. In this study, all RRIs tested showed the ability to improve the efficacy of ABC in the MAIDS model by reducing splenomegaly, hypergammaglobulinemia, and splenic proviral DNA levels.

Keywords: Ribonucleotide reductase; Murine AIDS; Hydroxyurea; Trimidox; Didox; Abacavir

1. Introduction

The need for alternative therapies for HIV infection has been highlighted by the demonstration of the development of multi-drug resistant HIV-1 strains (Shafer et al., 1998), metabolic abnormalities (Vigouroux et al., 1999), and the demonstration of low-level viral replication (Dornadula et al., 1999) in patients receiving highly active anti-retroviral therapy (HAART). These problems are further compounded by complicated drug administration schedules, often resulting in poor compliance, and by the high cost of therapy.

One alternative therapeutic strategy that has received attention is the use of ribonucleotide reductase inhibitors (RRI). Ribonucleotide reductase (RR) catalyses the formation of deoxyribonucleotides from the corresponding ribonucleotides, and represents the rate-limiting step in the production of DNA precursors (Reichard, 1993). Viruses, including HIV, are completely lacking in their ability to synthesize dNTPs, and are therefore completely dependent upon their host cells for production of these essential substances. Selective depletion of host cell dNTP pools without irreversibly impairing host cell dNTP synthesis is an intriguing target for inhibiting HIV replication (Mills et al., 1992a,b).

The RR inhibitor HU has been the primary focus of this strategy, since it is available for therapeutic clinical use, successfully inhibiting in vitro HIV-1 proviral DNA synthesis (Gao et al., 1994), and potentiating the anti-retroviral activity of several dideoxynucleoside (ddN) analogs (Gao et al., 1994; Malley et al., 1994; Lori et al., 1994). In addition, hydroxyurea has been shown to potentiate the anti-herpesvirus activity of several ddN analogs (Neyts and De Clerq, 1999).

* Corresponding author. Tel.: +1-859-323-1100x80479; fax: +1-859-323-1058.
E-mail address: Vsgall1@uky.edu (V.S. Gallicchio).

0166-3542/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2003.11.007

Clinical studies have supported the in vitro observations that HU can successfully potentiate the activity of ddI (Lori et al., 1997a,b), or ddI and d4T (Rutschmann et al., 2000; Galpin et al., 1998) in HIV-infected patients. It has also been shown that regimens containing a protease inhibitor, an NRTI, and HU are successful in reducing viral load to undetectable levels, decrease the number of activated CD8+ cells, and increase the number of CD4+ cells (Lori et al., 1999a,b).

The widespread use of HU in HIV-infected patients is still somewhat controversial due to its notorious ability to induce significant hematological disturbance (Maserati, 1999). In patients with advanced disease or in those receiving other anti-retroviral drugs that suppress hematopoiesis, the bone marrow suppression associated with HU may be further exacerbated (Foli et al., 1997). In addition to these problems, the rapid plasma clearance of HU (Moore and Hurlbert, 1985) and its relatively weak in vitro enzyme inhibition (Elford, 1968) may limit the in vivo RR inhibitory activity of HU. In an attempt to achieve greater RR inhibition than HU, several more potent RR inhibitors have been synthesized (van't Riet et al., 1979). Two of these compounds TX and DX were found to be particularly potent in vitro RR inhibitors (Elford and van't Riet, 1985). The structures of TX, DX, and HU are shown in Fig. 1.

A number of differences have been observed between the novel RRIs DX and TX in comparison to HU. Potentially the most important difference from a clinical perspective may be the effects DX and TX have upon dNTP levels compared to HU (Elford and van't Riet, 1985). HU has been consistently shown to reduce only the purine dNTP pools (dATP and dGTP) in various cell lines (Snyder et al., 1984; Bianchi et al., 1986; Gao et al., 1998). In contrast, DX and TX have been shown to reduce pools of both purine and pyrimidine dNTPs (Elford et al., 1980; Szekeres et al., 1994a; Elford and van't Riet, 1985). These findings may prove to be of clinical importance by allowing a larger number of therapeutic options in treatment of human AIDS.

Additional studies have shown that TX and DX have more potent in vivo anti-tumor activity than HU in several murine tumor models (Szekeres et al., 1994a,b; Elford et al., 1979). Results from our laboratory and others have shown that TX and DX have effective anti-retroviral activity both alone and in combination with ddI in the MAIDS model (Mayhew et al., 1997), HIV-infected HuPBMC SCID model (Ussery et al., 1999), and the Rauscher murine retrovirus model (Mills et al., 1992a,b). Importantly, it has also been demonstrated that TX and DX are less toxic to normal human and murine hematopoietic progenitor cells when compared to HU in vitro and in vivo (Mayhew et al., 1999, 2002b).

MAIDS is induced by inoculation with a complex of retroviruses called LPBM5 murine leukemia virus (MuLV), originally recovered from a radiation-induced lymphoma of C57BL/6 (B6) mice (Laterjet and Duplan, 1962). Many of the features of MAIDS are similar to those of HIV. Some of these similarities include development of a profound immunodeficiency characterized by deficits in B- and T-lymphocyte function as well as deficiencies in macrophage function. Early stage disease is characterized by polyclonal activation of lymphocytes and proliferation associated with progressive lymphadenopathy and splenomegaly (Chattopadhyay et al., 1991; Jolicoeur, 1991). Advanced stages of the disease are associated with profound immunodeficiency and enhanced susceptibility to opportunistic infections (Doherty et al., 1995) and development of secondary neoplasms, especially B-cell lymphomas (Buller et al., 1987).

Despite the similarities between HIV and MAIDS, there are several important differences. The major cellular targets for LPBM5 MuLV infection are B-lymphocytes, and not CD4 T cells as in HIV. LPBM5 MuLV is also much simpler in structure than HIV, lacking the tat, rev, and nef regulatory genes (Magnani et al., 1997). Also, the cause of death in MAIDS is believed to be severe pulmonary compromise secondary to lymphoid infiltration and enlarged thoracic lymph nodes (Jolicoeur, 1991).

MAIDS has been widely used as a model to evaluate experimental anti-HIV-1 compounds (Suruga et al., 1998; Fraternale et al., 2002; Magnani et al., 1997; Mayhew et al., 2002a). A particular advantage with the use of MAIDS for evaluation of experimental anti-retroviral compounds is that, unlike other murine models of retrovirus infection, the disease progresses over a substantial period of time. This permits administration of experimental drugs for several months, and evaluations of therapeutic benefit versus toxicity can be made in infected animals. Because B-cell proliferation is a major component of LPBM5 MuLV infection, MAIDS provides a good model for the preclinical study of compounds such as HU that have been proposed to be beneficial in HIV infection due in part to their cytostatic properties (Lori and Lisziewicz, 1998).

It has previously been demonstrated by our laboratory that the RRIs TX, DX, and HU serve as effective treatments for LPBM5 MuLV infection alone (Mayhew et al., 1997, 1999),

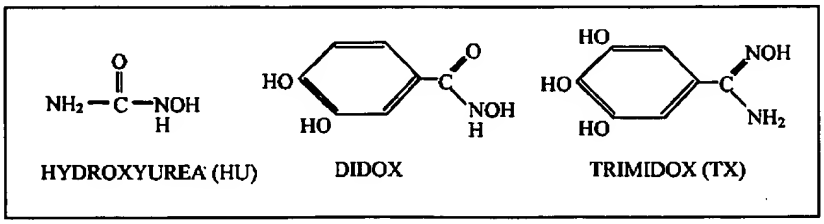

Fig. 1. Structure of RRIs.

or in combination with ddI (Mayhew et al., 1997). It has also been demonstrated that HU, TX, and DX can safely be administered to MAIDS infected mice for over 1 year (Mayhew et al., 1997). Further comparisons of TX and DX with HU have shown that TX and DX are less toxic to normal and infected bone marrow than HU (Mayhew et al., 1997). Most recently it has been demonstrated that short-term treatment with TX and DX is able to reverse late-stage MAIDS disease (Mayhew et al., 2002a). The purpose of the described studies was to further compare in vivo the ability of hydroxyurea and the novel ribonucleotide reductase inhibitors DX and TX to suppress retroviral infection in the MAIDS model when combined with the NRTI compound abacavir.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice aged 8–10 weeks were purchased from Charles River/NCI (Bethesda, MD, USA), and were housed in micro-isolator cages in a temperature and humidity-controlled environment. Mice were fed Purina Lab Chow and water ad libitum. The experimental animal protocol used in these studies was approved by the University of Kentucky IACUC committee.

2.2. Infection of mice with LPBM5 MuLV

The G6 subclone of chronically LPBM5 MuLV-infected SC-1 cells was kindly provided by Dr. Donald Cohen, Department of Microbiology and Immunology, University of Kentucky. G6 cells were grown in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO, USA), and 1% penicillin–streptomycin (Gibco). Virus used for inoculation of mice was prepared by removal of the cell supernatant. The cell monolayer was subsequently lysed by repeated freeze–thaw cycles to yield intra-cellular virus (Mayhew et al., 1997, 2002a,b). This was combined with the cell supernatant, centrifuged at 300 × *g* for 10 min and 0.45 μm filtered. Inoculation of virus was performed by two separate 0.5 ml i.p. injections 3 days apart, per mouse.

2.3. Treatment of LPBM5 MuLV-infected mice

LPBM5-infected animals were randomly assigned to groups of eight to receive the various treatment regimens (see Table 1). Doses of RRIs were chosen based upon concentrations, which had previously been shown by our laboratory to enhance the activity of ddI (Mayhew et al., 1997), and doses of ABC were chosen based upon the manufacturer's recommendations. TX and DX were provided by Dr. Howard Elford (Molecules for Health Inc., Richmond, VA, USA). HU was purchased from Sigma Chemical Co., and ABC was provided by Dr. Trevor Scott (Glaxo Smith Kline, Research Triangle Park, NC, USA).

Table 1
Experimental groupings

Normal control	Infected + TX150
Infected control (MC)	Infected + TX150 + ABC100
Infected + HU60	Infected + TX150 + ABC200
Infected + HU60 + ABC200	Infected + DX300
Infected + HU150	Infected + DX300 + ABC200
Infected + HU150 + ABC200	Infected + DX350
Infected + HU200	Infected + ABC200

All doses of drugs are in milligram per kilogram of body weight.

All mice in groups listed above received the listed drug doses in milligram per kilogram body weight. In addition to the groups listed above, 16 non-infected, non-treated animals served as normal controls, and 16 LPBM5-infected, non-treated animals served as MAIDS controls. Drug treatment began 7 days after the first inoculation of virus, and continued daily for 8 weeks. HU, TX, DX, and ABC were injected i.p. in a final volume of 0.5 ml for each drug. Groups receiving combination therapy were given a total of two injections per day (0.5 ml of each drug). Drugs were prepared weekly, sterile filtered, and stored at 4 °C. Drugs were warmed to body temperature prior to injection.

2.4. Procurement of tissues for analysis of anti-viral drug efficacy and hematopoietic toxicity

After 4 and 8 weeks of treatment, four mice from each group were randomly selected and sacrificed for analysis of anti-viral drug efficacy. Blood was collected from each mouse by cardiac puncture, and complete blood counts were performed using a Baker 9110 +CP (ABX Diagnostics, Irvine, CA, USA). Serum was obtained by centrifugation of blood at 10,000 × *g* for 10 min and was frozen at −20 °C until used. The spleens were rapidly removed, weighed, and cut approximately in half. One half was immediately frozen in a dry-ice/ethanol bath and stored at −80 °C until used for DNA extraction.

2.5. Enzyme-linked immunosorbant assay for murine IgG

Serum was obtained after 4 and 8 weeks as described above and stored at −20 °C until used. A standard enzyme-linked immunosorbent assay technique (ELISA) was used to quantify levels of murine IgG. Ninety-six-well microtiter plates were coated overnight at 4 °C with 1.5 μg/ml goat anti-mouse IgG (Fc-chain-specific; Jackson ImmunoResearch, West Grove, PA, USA) in 0.1 M $NaHCO_3$. After washing and blocking with 0.1% bovine serum albumin (Sigma) in PBS, diluted serum samples and serially diluted murine IgG standards (Sigma) of known concentrations were added to plates and incubated

at room temperature for 2 h. After washing, 0.3 μg/ml alkaline phosphatase-conjugated anti-mouse IgG (Jackson ImmunoResearch) in PBS was added and incubated at room temp for 1 h. After washing, *p*-nitrophenyl phosphate substrate (Sigma) was added and incubated for 15 min at 37 °C. The reaction was stopped by addition of 3 M NaOH and the absorbance of each well was read at 405 nm. Serum IgG concentrations were determined by comparison with the murine IgG standards.

2.6. Extraction of genomic DNA

Genomic DNA was extracted from three spleens from each experimental group using the DNeasy tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Spleen size was not taken into account when selecting tissue for DNA extraction. DNA was eluted from spin columns and stored at −20 °C until used in PCR reactions. The yield and purity of extracted DNA was quantified spectrophotometrically.

2.7. Semi-quantitative PCR for integrated proviral DNA

The following primers, specific for the p12 gag region of the LPBM5 defective virus (BM5-def) genome were used for PCR amplification: 5′-CCT TTT CCT TTA TCG ACA CT-3′ (sense), and 5′-ACC AGG GGG GGA ATA CCT CG-3′ (anti-sense). The expected size of the amplified product was 246 base pairs. A second pair of oligonucleotide primers, designed using the Oligo (version 6.15) computer software (Molecular Biology Insights Inc., Cascade, CO, USA) were used to amplify a region of the murine B-actin gene which served as an endogenous control. The B-actin primer sequences are: 5′-CAC TGT GCC CAT CTA CGA-3′ (sense), and 5′-ACA GGA TTC CAT ACC CAA G-3′ (anti-sense) and amplified a product of 334 base pairs. PCR amplification in the linear range was performed in a final volume of 25 μl, containing 0.25 μg genomic DNA, 1 pg of each primer and a 1× solution of AmpliTaq gold master mix (Applied Biosystems, Branchburg, NJ, USA). BM5def was amplified by hot-start and initial denaturation at 95 °C for 10 min followed by 22 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 1.5 min and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 8 min. β-Actin was amplified by hot-start and initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 45 s, and extension at 72 °C for 45 s followed by a final extension at 72 °C for 10 min. For quantification, gels were stained with ethidium bromide and densitometry was performed using 1D Image Analysis Software (Kodak, Rochester, NY, USA). Each BM5def band was normalized to its corresponding G6PDH band before comparison between experimental groups, and each data point represents the mean and S.D. for a minimum of three mice per experimental group.

2.8. Assay of bone marrow progenitors

Contents of femurs were flushed in ice cold PBS with a 22-gauge needle. Single cell suspensions of bone marrow were made by gentle flushing through an 18-gauge needle, and bone marrow from each experimental group was pooled for analysis of committed granulocyte/macrophage (CFU-GM), and erythroid (BFU-E) progenitors, according to previously published methods (Mayhew et al., 1997). Briefly, 25,000 bone marrow cells were plated in duplicate in 1 ml methylcellulose medium (Stem Cell Technologies, Vancouver, Canada) containing 1% methylcellulose in Iscove's minimal Dulbecco's media, 15% FBS, 1% bovine serum albumin, 10 μg bovine pancreatic insulin, 200 μg/ml human transferrin (iron saturated), 10^{-4} M β-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml recombinant murine (rm) stem cell factor, 10 ng/ml recombinant human (rh) interleukin-6, and 3 units/ml rh-erythropoietin. After vortexing, duplicate samples were plated in six-well tissue culture plates and incubated at 37 °C in air containing 5% CO_2 for 10 days. Using an inverted microscope, CFU-GM and BFU-E were identified based on their morphology, and colonies containing greater than 50 cells were scored.

2.9. Statistical analysis

The Student's two-tailed *t*-test was used to determine the significance of differences between groups. A *P*-value of <0.05 was considered to be significant.

3. Results

3.1. Inhibition of splenomegaly

Infection of mice with LPBM5 MuLV results in immunodeficiency disease and lymphoproliferation characterized by splenomegaly (Morse et al., 1992). In this study all infected control animals developed extensive peripheral lymphadenopathy and splenomegaly. Spleen weights from each experimental group are shown in Fig. 2. By the end of the eighth week of infection, spleens of infected controls weighed approximately six- to seven-fold higher than non-infected controls. ABC monotherapy showed only a modest reduction in spleen weight versus MAIDS controls (MC) (approximately 29%). Combination of ABC with RRIs was able to greatly improve the activity level of ABC, reducing the increase in spleen weight due to MAIDS infection by: 86% for HU150 + ABC200 and approximately 90% for TX150 + ABC200; 91% for DX300 + ABC200. The combination treatment group spleen weights were only slightly different from normal control values. The best anti-viral activity, however, was demonstrated by DX350 monotherapy, which was not significantly different from non-infected control values ($P = 0.347$), but was significantly different from MC ($P < 0.005$).

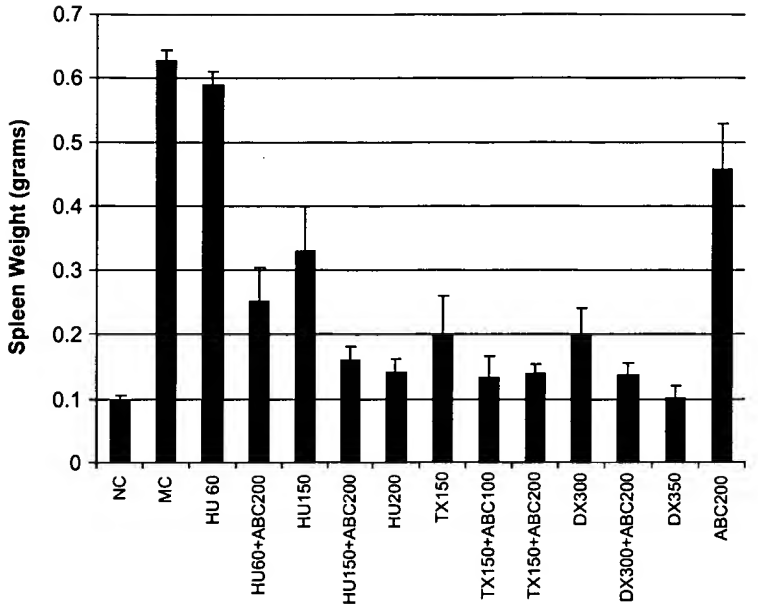

Fig. 2. Effect of drug treatment on inhibition of viral-induced splenomegaly in LPBM5 MuLV-infected mice. LPBM5 MuLV-infected animals were treated daily for 8 weeks with RRIs alone or in combination with ABC. After 8 weeks of drug treatment, animals were sacrificed and their spleens were weighed. Bars represent the mean and standard deviation of at least three animals per group.

3.2. *Inhibition of hypergammaglobulinemia*

Early progression of MAIDS is associated with polyclonal activation of B-cells resulting in increased serum immunoglobulin concentrations (Klinman and Morse, 1989). Mean serum IgG concentrations for each experimental group are shown in Fig. 3. By the end of the eighth week of drug treatment, hypergammaglobulinemia was evident in infected controls, which showed a six- to seven-fold increase in IgG levels versus non-infected controls. All drug treatment groups were effective in significantly lowering the level of serum immunoglobulin versus infected control, with the exception of the lowest monotherapeutic dose of HU. ABC200 showed approximately 45% inhibition, with all HU and TX regimens showing comparable results. The most effective treatment at inhibiting the increase in serum IgG level was in the DX300 + ABC200 group. This group showed no increase in IgG levels versus non-infected controls.

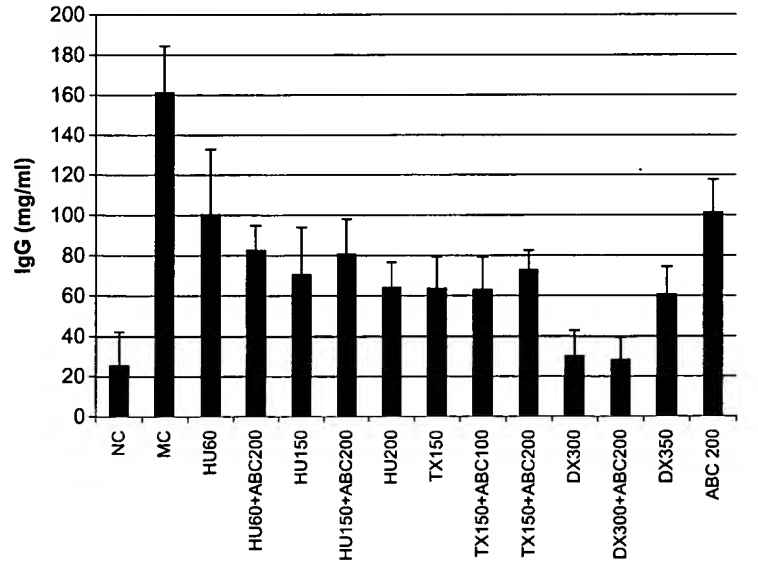

Fig. 3. Effect of drug treatment on inhibition of viral-induced hypergammaglobulinemia in LPBM5 MuLV-infected mice. LPBM5 MuLV-infected animals were treated daily for 8 weeks with RRIs alone or in combination with ABC. After 8 weeks of drug treatment, animals were sacrificed and their serum IgG levels were measured as described in the Section 2. Bars represent the mean and standard deviation of at least three animals per group.

DX300 monotherapy showed similar results, with a slightly higher mean value and slightly more variation within the group.

3.3. Inhibition of LPBM5 proviral DNA

Relative levels of splenic BM5 def proviral DNA were evaluated by semi-quantitative PCR (Fig. 4A and B). After 8 weeks of drug treatment, all drug treated groups showed reduced levels of pro-viral DNA compared to infected controls.

3.4. Gross toxicity

There was no evidence of gross toxicity in any of the treatment groups presented in this paper, however, when HU200 mg/kg was combined with ABC, fatal toxicity was observed in a high percentage of animals receiving these regimens (data not shown). This is in contrast to TX and DX, which were well tolerated at relatively high doses both alone and in combinations with ABC.

3.5. Effects on peripheral blood indices

Complete blood counts were performed after 8 weeks of drug treatment to monitor the effects of drug treatment on peripheral blood indices. Although no statistically significant changes in white blood count (WBC) values were observed, there was a general trend present indicating that groups treated with HU had the lowest mean WBC, followed by DX, then TX. ABC treatment did not tend to alter WBC appreciably (Fig. 5).

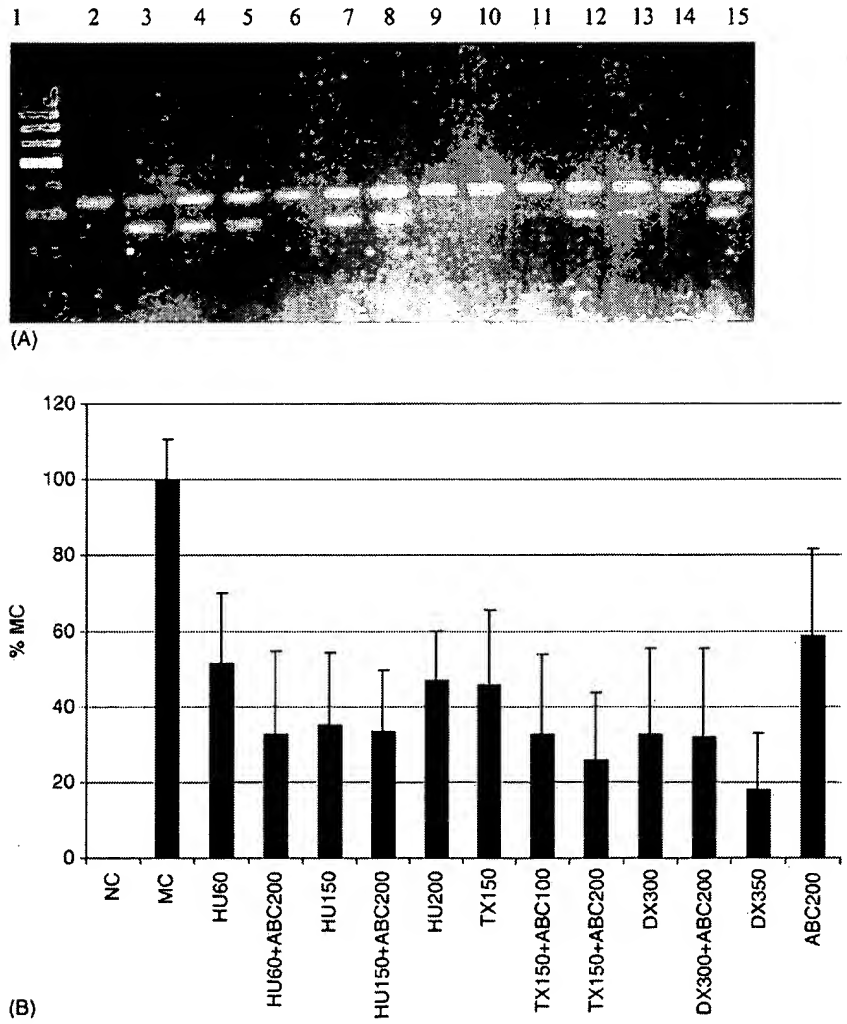

Fig. 4. (A) Effects of drug treatment on levels of splenic proviral DNA in LPBM5 MuLV-infected mice. The top row of bands represents β-actin, and the bottom row of bands represents BM5-def. Lane 1: molecular weight marker; lane 2: NC; lane 3: MC; lane 4: HU60; lane 5: HU60 + ABC200; lane 6: HU150 + ABC200; lane 7: HU200; lane 8: TX150; lane 9: TX150 + ABC100; lane 10: TX150 + ABC200; lane 11: DX300; lane 12: DX300 + ABC200; lane13: DX350; lane 14: DX350 + ABC200; lane 15: ABC200. The bands above show a representative example from each experimental group. (B) Effects of drug treatment on levels of splenic pro-viral DNA in LPBM5 MuLV-infected mice. Densitometry readings from semi-quantitative PCR are represented here as percentages of the infected control animals. Bars represent the mean and standard deviation of three mice per group.

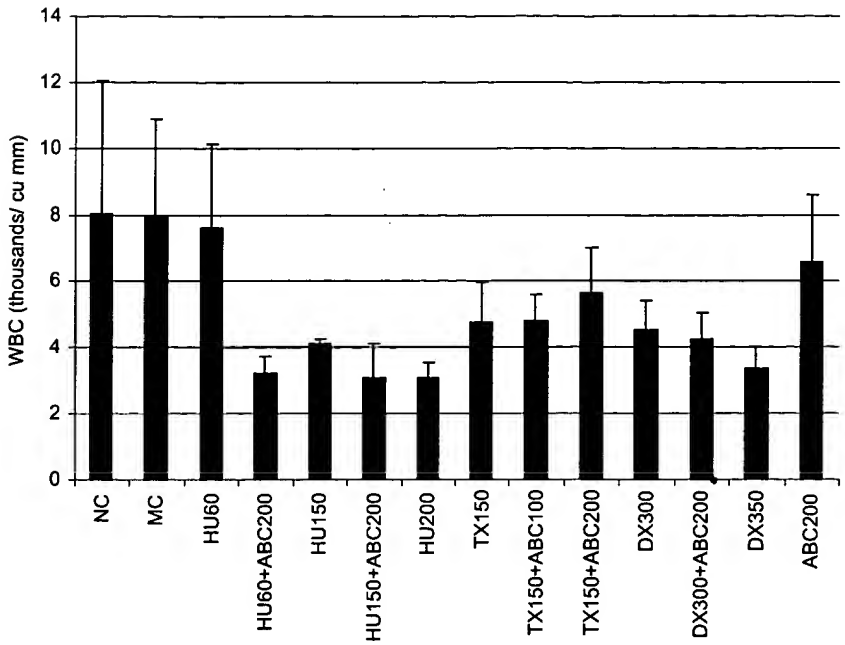

Fig. 5. Effects of drug treatment on WBC in LPBM5 MuLV-infected mice. WBC is expressed as thousands of cells per cubic millimeter. Bars represent the mean and standard deviation of at least three mice per group.

In the most effective anti-viral HU regimens (HU200; HU150 + ABC200), the red blood cell count (RBC) was significantly lower than the RBC from MC. Treatment with DX350 also significantly reduced the RBC values compared to MC, but no other regimen produced significant alterations in the RBC values.

The most effective anti-viral regimens containing HU (HU200; HU150 + ABC200) significantly lowered hematocrit (HCT) values compared to infected controls. No other therapeutic regimen significantly altered HCT values compared to infected controls.

HU200 and DX350 produced platelet count values that were significantly lower than non-infected control values, but they were not significantly lower than infected control values.

3.6. Effects of drug treatment on hematopoietic progenitor cells

Drug treatment regimens containing HU150, HU150 + ABC200, and HU200 produced significantly low numbers of femoral CFU-GM colonies compared to infected control values, which were significantly lower than non-infected control values. No other treatment groups lowered the femoral CFU-GM, and in fact, regimens containing DX actually increased the numbers of CFU-GM colonies compared to non-infected controls. This is also true of ABC monotherapy (Fig. 6).

No significant effects of femoral BFU-E colony numbers were observed in any drug treatment groups (data not shown).

4. Discussion

The cellular enzyme RR has been determined to be the rate-limiting step in de novo synthesis of dNTPs (Thelander and Reichard, 1979). Inhibition of RR by HU results in a reduction of dNTP pools, followed by a cessation in DNA synthesis and arrest of the cell cycle in S-phase (Moore and Hurlbert, 1985). HU has also been shown to cause cell death when used at high doses, or for prolonged exposures (Moore and Hurlbert, 1985). These properties of HU make it an interesting compound with potential benefits for HIV-infected patients. For example, the reduction of dNTP pools by HU may have an effect on HIV replication because HIV has been demonstrated to be extremely sensitive to fluctuations in dNTP levels (Gao et al., 1993; Meyerhans et al., 1994). The effects of HU upon dNTP levels also allows the possibility that HU could enhance the activity of nucleoside or -tide analog RT inhibitors because the nRTIs are structural analogs of the physiological dNTPs (Sommadossi, 1993), and compete with those physiological dNTPs for binding to the RT enzyme of HIV. It has been established that the activity of nRTIs is not completely dependent upon the actual level of nRTI, but lies mainly in the level of nRTI to its physiological counterpart (Johns and Gao, 1998). Therefore, reducing the endogenous level of dNTP may prove to be as effective as increasing the level of nRTI (Johns and Gao, 1998). At this point, HU has only been demonstrated to most consistently potentiate the effects of adenosine analogs (particularly ddI), with lesser potentiation of thymidine, cytosine, and guanosine analogs (Gao et al., 1994; Lori et al., 1994). In contrast to HU, TX and DX have been demonstrated to

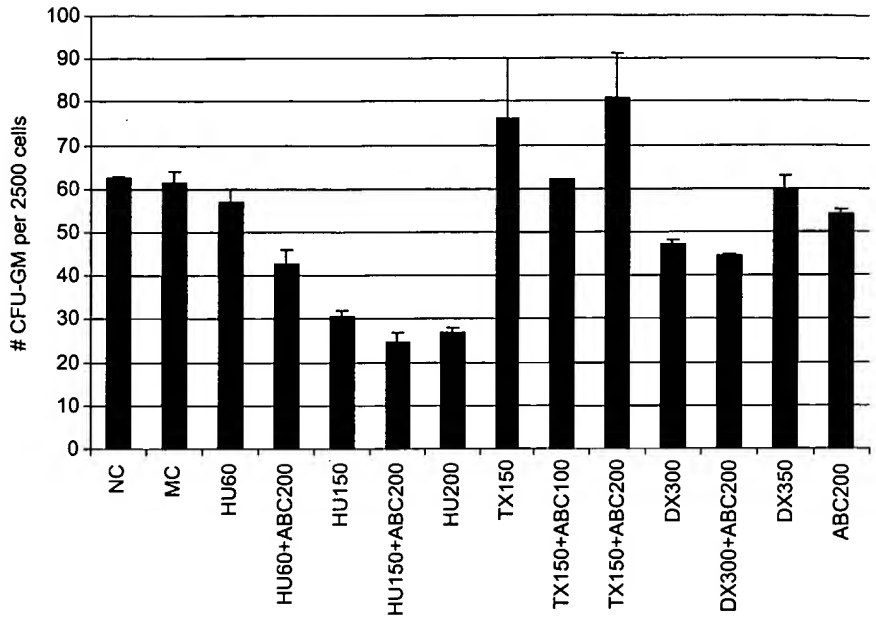

Fig. 6. Effects of drug treatment on bone marrow progenitor cells (CFU-GM) in LPBM5 MuLV-infected mice. The numbers of progenitor colonies (CFU-GM) are represented here as the number of colonies per 25,000 femur cells plated as described in the Section 2. Bars represent the mean and standard deviation of duplicate counts of pooled bone marrow from each group.

significantly reduce not only the dATP pool, but also the dCTP and dGTP pools in various cell lines (Elford and van't Riet, 1985; Szekeres et al., 1994a; Tihan et al., 1991). Based on this information our group speculated that TX and DX may have the ability to increase the anti-HIV-1 activity of a larger number of ddNs than HU, potentially providing more therapeutic options in the treatment of human AIDS.

The data presented here clearly indicate that the RRIs HU, TX, and DX are effective treatments with the ability to influence several parameters associated with disease progression in the murine AIDS model of retrovirus infection and are able to improve the activity of the guanine-like RT inhibitor ABC in the murine AIDS model without inducing significant hematological toxicity.

RRI doses used in this study were based on concentrations previously demonstrated by our laboratory to enhance the activity of ddI (Mayhew et al., 1997) and doses of ABC were chosen based upon recommendations by the manufacturer and upon the results of a 24 h maximum tolerated dose experiment (data not shown). Notable observations made in this study were that all RRIs provided effective inhibition of viral-induced disease pathophysiology, however, the use of HU while highly effective is associated with perturbations of the hematological system. This is in contrast to TX and DX, which were similarly effective, but induced far fewer perturbations to the hematological system than HU. This observation is confirmed by other studies from our laboratory (Mayhew et al., 1997, 1999, 2002b). Of particular interest was the observation that all RRIs could safely be combined with ABC, improving the activity of ABC in the model, as well as demonstrating that ABC improves the activity of sub-optimal doses of the RRIs. Although the improvement of RRI doses by the addition of ABC to the regimen was not significantly different from RRI monotherapy, general trends were clearly visible, however, statistical significance was difficult to obtain due to the high level of efficacy demonstrated by RRI monotherapy. Given the level of activity and the lack of significant toxicity observed in this study with TX and DX when combined with ABC, and the recent demonstration by Mayhew et al. (2002a) that TX and DX can reverse late-stage disease pathology, it would be of interest to administer these combination regimens to animals with late-stage infection.

With respect to the measurements of hematological toxicity, HU and DX were both associated with lowered WBC values compared to non-infected and infected controls, however, HU significantly depleted the number of committed hematological progenitor cells in the bone marrow compartment. This is in contrast to DX. DX actually increased the numbers of bone marrow progenitors. At the doses of TX used, no hematological perturbations were observed when TX was used alone or in combination with ABC.

LPBM5 MuLV viremia was not directly measured in this experiment, but it was indirectly measured by evaluation of levels of proviral DNA in spleens of infected animals treated with RRIs. All RRIs showed potent activity at reducing the levels of proviral DNA, with little difference in activity among groups. The main difference in treatment among groups is that the most effective regimens containing HU were associated with hematological toxicity as reflected in the lower peripheral blood and bone marrow indices.

In addition to these findings, previous experiments by our group in the HuPBMC SCID model of HIV-1 infection have demonstrated that TX and DX are able to reduce HIV-1 RNA titers in vivo (Broud et al., 1998; Ussery et al., 1999). Therefore, TX and DX may have a direct inhibitory effect upon HIV-1 replication if administered to infected individuals.

HIV-1 replication is characterized by T-cell activation, with the most efficient virus replication occurring in activated T cells (Stevenson et al., 1990). In HIV-1 infection, CD8+ cells increase dramatically (Giorgi et al., 1993), and CD8+ cells have been implicated in the immunopathology of HIV infection (Zinkernagel and Hengartner, 1994). RRIs TX and DX may have the same beneficial effects as HU upon CD4+ and CD8+ cell activation (Lori, 1999). In support of this idea is the observation that TX and DX inhibited virus induced B-lymphocyte activation and proliferation (characterized by hypergammaglobulinemia and splenomegaly) in the MAIDS model.

In conclusion, the ability of the RRIs HU, TX, and DX to improve the activity of ABC in the MAIDS model was comparatively examined. All three RRIs showed the ability to improve the activity of ABC in the MAIDS model, as determined by inhibition of splenomegaly, hypergammaglobulinemia, and proviral DNA levels, however, in the most effective regimens containing HU, disturbances in hematological function were observed. This is in stark contrast to TX and DX, which showed similar potency to HU containing regimens, but with little or no disturbance in hematological function at the doses examined. In light of these observations, it is possible that TX and DX may prove to be of benefit to HIV-1-infected individuals when combined with current treatment regimens.

Acknowledgements

The authors wish to acknowledge the assistance of Dr. Trevor Scott (Glaxo Smith Kline, Research Triangle Park, NC, USA) for graciously providing ABC. These studies were supported by NIH grant R44-AI36095 awarded to H.L.E.

References

Bianchi, V., Pontis, E., Reichard, P., 1986. Changes of deoxyribonucleoside triphosphates pools induced by hydroxyurea and their relation to DNA synthesis. J. Biol. Chem. 261 (34), 16037–16042.

Broud, D., Piper, J., Gallicchio, V., Black, P., Kunder, S., Goldberg, H., Elford, H., Hall, B., van't Riet, B., Bacho, M., Mayhew, C., Papermaster, S., Oakley, O., Ussery, M., 1998. Novel ribonucleotide reductase (RR) inhibitors, didox and trimidox, produce antiretroviral effects in the murine acquired immunodeficiency (MAIDS) and in the HIV-infected HuPBMC SCID models. Antiviral Res. 37, A58 (Abstract).

Buller, R.M., Yetter, R.A., Fredrickson, T.N., Morse III, H.C., 1987. Abrogation of resistance to severe mousepox in C57BL6 mice infected with LP-BM5 murine leukemia viruses. J. Virol. 61 (2), 383–387.

Chattopadhyay, S.K., Sengupta, D.N., Fredrickson, T.N., Morse, H.C., Hartley, J.W., 1991. Characteristics and contributions of defective, ecotropic, and mink cell focus-inducing viruses involved in a retrovirus-induced immunodeficiency syndrome of mice. J. Virol. 65 (8), 4232–4241.

Doherty, T.M., Morse, H.C., Coffman, R.L., 1995. Modulation of specific T cell responses by concurrent infection with Leishmania major and LP-BM5 murine leukemia viruses. Int. Immunol. 7 (1), 131–138.

Dornadula, G., Zhang, H., Van Uitert, B., Stern, J., Livornese Jr., L., Ingerman, M.J., Witek, J., Dedanis, R.J., Natkin, J., DeSimone, J., Pomerantz, R.J., 1999. Residual HIV-1 RNA in blood plasma of patients taking suppressive highly active antiretroviral therapy. J. Am. Med. Assoc. 282, 1627–1632.

Elford, H.L., 1968. Effect of hydroxyurea on ribonucleotide reductase. Biochem. Biophys. Res. Commun. 33 (1), 129–135.

Elford, H.L., van't Riet, B., 1985. Inhibition of nucleoside diphosphates reductase by hydroxybenzohydroxamic acid derivatives. Pharmacol. Ther. 29 (2), 239–254.

Elford, H.L., Wampler, G.L., van't Riet, B., 1979. New ribonucleotide reductase inhibitors with antineoplastic activity. Cancer Res. 39 (3), 844–851.

Elford, H.L., van't Riet, B., Wampler, G.L., Lin, A.L., Elford, R.M., 1980. Regulation of ribonucleotide reductase in mammalian cells by chemotherapeutic agents. Adv. Enzyme Regul. 19, 151–168.

Foli, A., Lori, F., Maserati, R., Tinelli, C., Minoli, L., Lisziewicz, J., 1997. Hydroxyurea and didanosine as a more potent combination than hydroxyurea and zidovudine. Antivir. Ther. 2 (1), 33–40.

Fraternale, A., Cassabianca, A., Orlandi, C., Chiarantini, L., Brandi, G., Silvestri, G., Magnani, M., 2002. Repeated cycles of alternate administration of fludarabine and zidovudine plus didanosine inhibits murine AIDS and reduces proviral DNA content in lymph nodes to undetectable levels. Virology 302 (2), 354–362.

Galpin, J.E., Lori, F., Globe, D.R., Casciato, D., 1998. Safety, sheltering, & synergy of hydroxyurea (HU) with ddI or ddI plus d4T in HIV-infected patients. Fifth Conf. Retrovir. Oppor. Infect. (abstract no. 645).

Gao, W.Y., Cara, A., Gallo, R.C., Lori, F., 1993. Low levels of deoxynucleotides in peripheral blood lymphocytes: a strategy to inhibit human immunodeficiency virus type 1 replication. Proc. Natl. Acad. Sci. U.S.A. 90 (19), 8925–8928.

Gao, W.Y., Johns, D.G., Mitsuya, H., 1994. Anti-human immunodeficiency virus type 1 activity of hydroxyurea in combination with 2′,3′-dideoxynucleosides. Mol. Pharmacol. 46 (4), 767–772.

Gao, W.Y., Zhou, B.S., Johns, D.G., Mitsuya, H., Yen, Y., 1998. Role of the M2 subunit of ribonucleotide reductase in regulation by hydroxyurea of the activity of the anti-HIV-1 agent 2′,3′-dideoxyinosine. Biochem. Pharmacol. 56 (1), 105–112.

Giorgi, J.V., Liu, Z., Hultin, L.E., Cumberland, W.G., Hennessey, K., Detels, R., 1993. Elevated levels of CD38+ CD8+ T cells in HIV infection add to the prognostic value of low CD4+ T cell levels: results of 6 years of follow-up. The Los Angeles Center, Multicenter AIDS Cohort Study. J. Acquir. Immune. Defic. Syndr. 6 (8), 904–912.

Johns, D.G., Gao, W.Y., 1998. Selective depletion of DNA precursors: an evolving strategy for potentiation of dideoxynucleoside activity against human immunodeficiency virus. Biochem. Pharmacol. 55 (10), 1551–1556.

Jolicoeur, P., 1991. Murine acquired immunodeficiency syndrome (MAIDS): an animal model to study the AIDS pathogenesis. FASEB J. 5 (10), 2398–2405.

Klinman, D.M., Morse, H.C., 1989. Characteristics of B cell proliferation and activation in murine AIDS. J. Immunol. 142 (4), 1144–1149.

Laterjet, R., Duplan, J.F., 1962. Experiment and discussion on leukaemogenesis induced cell free extracts of radiation-induced leukaemia in mice. Int. J. Radiat. Biol. 5, 339–344.

Lori, R., 1999. Hydroxyurea and HIV: 5 years later-from antiviral to immune-modulating effects. AIDS 13, 1422–1433.

Lori, F., Lisziewicz, J., 1998. Hydroxyurea: mechanisms of HIV-1 inhibition. Antivir. Ther. 3 (Suppl. 4), 25–33.

Lori, F., Malykh, A., Cara, A., Sun, D., Weinstein, J.N., Lisziewicz, J., Gallo, R.C., 1994. Hydroxyurea as an inhibitor of human immunodeficiency virus type 1 replication. Science 266 (5186), 801–805.

Lori, F., Jessen, H., Foli, A., Lisziewicz, J., Matteo, P.S., 1997a. Long-term suppression of HIV-1 by hydroxyurea and didanosine. JAMA 277 (18), 1437–1438.

Lori, F., Malykh, A., Foli, A., Maserati, R., De Antoni, A., Minoli, L., Padrini, D., Antoni, A., Barchi, E., Jessen, H., Wainberg, M.A., Gallo, R.C., Lisziewicz, J., 1997b. Combination of a drug targeting the cell with a drug targeting the virus controls human immunodeficiency virus type 1 resistance. AIDS Res. Hum. Retroviruses 13 (16), 1403–1409.

Lori, F., Jessen, H., Lieberman, J., Clerici, M., Tinelli, C., Lisziewicz, J., 1999a. Immune restoration by combination of a cytostatic drug (hydroxyurea) and anti-HIV drugs (didanosine and indinavir). AIDS Res. Hum. Retroviruses 15 (7), 619–624.

Lori, F., Jessen, H., Lieberman, J., Finzi, D., Rosenberg, E., Tinelli, C., Walker, B., Siliciano, R.F., Lisziewicz, J., 1999b. Treatment of human immunodeficiency virus infection with hydroxyurea, didanosine, and a protease inhibitor before seroconversion is associated with normalized immune parameters and limited viral reservoir. J. Infect. Dis. 180 (6), 1827–1832.

Magnani, M., Fraternale, A., Casabianca, A., Schiavano, G.F., Chiarantini, L., Palamara, A.T., Ciriolo, M.R., Rotilio, G., Garaci, E., 1997. Antiretroviral effect of combined zidovudine and reduced glutathione therapy in murine AIDS. AIDS Res. Hum. Retroviruses 13 (13), 1093–1099.

Malley, S.D., Grange, J.M., Hamedi-Sangsari, F., Vila, J.R., 1994. Synergistic anti-human immunodeficiency virus type 1 effect of hydroxamate compounds with 2′,3′-dideoxyinosine in infected resting human lymphocytes. Prox. Natl. Acad. Sci. U.S.A. 91 (23), 11017–11021.

Maserati, R., 1999. Hydroxyurea in the treatment of HIV-1 infection: toxicity and side effects. J. Biol. Regul. Homeost. Agents 13 (3), 181–185.

Mayhew, C., Oakley, O., Piper, J., Hughes, N.K., Phillips, J., Birch, N.J., Elford, H.L., Gallicchio, V.S., 1997. Effective use of ribonucleotide reductase inhibitors (didox and trimidox) alone or in combination with didanosine (ddI) to suppress disease progression and increase survival in murine acquired immunodeficiency syndrome (MAIDS). Cell. Mol. Biol. 43 (7), 1019–1029.

Mayhew, C.N., Phillips, J.D., Greenberg, R.N., Birch, N.J., Elford, H.L., Gallicchio, V.S., 1999. In vivo and in vitro comparison of the short-term hematopoietic toxicity between hydroxyurea and trimidox or didox, novel ribonucleotide reductase inhibitors with potential anti-HIV-1 activity. Stem Cells 17 (6), 345–356.

Mayhew, C.N., Phillips, J.D., Cibull, M.L., Elford, H.L., Gallicchio, V.S., 2002a. Short-term treatment with novel ribonucleotide reductase inhibitors trimidox and didox reverses late-stage murine retrovirus-induced lymphoproliferativie disease with less bone marrow toxicity than hydroxyurea. Antivir. Chem. Chemother. 13 (5), 305–314.

Mayhew, C.N., Mampuru, L.J., Chendil, D., Ahmed, M.M., Phillips, J.D., Greenberg, R.N., Elford, H.L., Gallicchio, V.S., 2002b. Suppression of retrovirus-induced immunodeficiency disease (murine AIDS) by trimidox and didox: novel ribonucleotide reductase inhibitors with less bone marrow toxicity than hydroxyurea. Antiviral Res. 56 (2), 167–181.

Meyerhans, A., Vartanian, J.P., Hultgren, C., Plikat, U., Karlsson, A., Wang, L., Eriksson, S., Wain-Hobson, S., 1994. Restriction and enhancement of human immunodeficiency virus type 1 replication by modulation of intracellular deoxynucleoside triphosphate pools. J. Virol. 68 (1), 535–540.

Mills, D.L., Elford, H.L., van't Riet, B., 1992a. Didox and analogs are effective against retrovirus in a murine model. Antiviral Res. 17 (Suppl. 1), 76.

Mills, D.L., Elford, H.L., van't Riet, B., 1992b. Didox exhibits antiviral activity in a retrovirus animal model. Proc. Am. Assoc. Cancer. Res. 33, 399.

Moore, E.C., Hurlbert, R.B., 1985. The inhibition of ribonucleoside diphosphate reductase by hydroxyurea, guanazole and pyrazoloimidazole (IMPY). Pharmacol. Ther. 27 (2), 167–196.

Morse, H.C., Chattopadhyay, S.K., Makino, M., Fredrickson, T.N., Hugin, A.W., Hartley, J.W., 1992. Retrovirus-induced immunodeficiency in the mouse: MAIDS as a model for AIDS. AIDS 6 (7), 607–621.

Neyts, J., De Clerq, E., 1999. Hydroxyurea potentiates the antiherpesvirus activities of purine and pyrimidine nucleoside and nucleoside phosphonate analogs. Antimicrob. Agents Chemother. 43 (12), 2885–2892.

Reichard, P., 1993. From RNA to DNA, why so many ribonucleotide reductases? Science 260 (5115), 1773–1777.

Rutschmann, O.T., Vernazza, P.L., Bucher, H.C., Opravil, M., Ledergerber, B., Telenti, A., Malinverni, R., Bernasconi, E., Fagard, C., Leduc, D., Perrin, L., Hirschel, B., 2000. Long-term hydroxyurea in combination with didanosine and stuvadine for the treatment of HIV-1 infection. Swiss HIV Cohort Study. AIDS 14, 2145–2151.

Shafer, R.W., Winters, M.A., Plamer, S., Merigan, T.C., 1998. Multiple concurrent reverse transcriptase and protease mutations and multidrug resistance of HIV-1 isolates from heavily treated patients. Ann. Intern. Med. 128, 906–911.

Snyder, R.D., Wawra, E., Wintersberger, E., 1984. Deoxyribonucleoside triphosphates pools in human diploid fibroblasts and their modulation by hydroxyurea and deoxynucleosides: does hydroxyurea inhibit DNA replication in mouse cells by more than one mechanism? Biochem. Pharmacol. 33 (9), 1515–1518.

Sommadossi, J.P., 1993. Nucleoside analogs: similarities and differences. Clin. Infect. Dis. 16 (Suppl. 1), S7–S15.

Stevenson, M., Stanwick, T.L., Dempsey, M.P., Lamonica, C.A., 1990. HIV-1 replication is controlled at the level of T cell activation and proviral integration. EMBO J. 9 (5), 1551–1560.

Suruga, Y., Makino, M., Okada, Y., Tanaka, H., De Clercq, E., Baba, M., 1998. Prevention of murine AIDS development by (*R*)-9-(2-phosphonylmethoxypropyl)adenine. J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol. 18 (4), 316–322.

Szekeres, T., Fritzer, M., Strobl, H., Gharehbaghi, K., Findenig, G., Elford, H.L., Lhotka, C., Schoen, H.J., Jayaram, H.N., 1994a. Synergistic growth inhibitory and differentiating effects of trimidox and tiazofurin in human promyelocytic leukemia HL-60 cells. Blood 84 (12), 4316–4321.

Szekeres, T., Gharehbaghi, K., Fritzer, M., Woody, M., Srivastava, A., van't Riet, B., Jayaram, H.N., Elford, H.L., 1994b. Biochemical and antitumor activity of trimidox, a new inhibitor of ribonucleotide reductase. Cancer Chemother. Pharmacol. 34 (1), 63–66.

Thelander, L., Reichard, P., 1979. Reduction of ribonucleotides. Annu. Rev. Biochem. 48, 133–158.

Tihan, T., Elford, H.L., Cory, J.G., 1991. Studies on the mechanisms of inhibition of L1210 cell growth by 3,4-dihydroxybenzohydroxamic acid and 3,4-dihydroxybenzamidoxime. Adv. Enzyme Regul. 31, 71–83.

Ussery, M.A., Wood, O.L., Broud, D.D., Bacho, M.A., Kunder, S.C., Vona, S.F., Nielsen, C.J., Elford, H.L., 1999. Didox, a novel ribonucleotide reductase inhibitor, is more active than hydroxyurea in HIV-infected HuPBMC SCID mice. Antivir. Ther. 4 (Suppl. 1), 12 (abstract).

van't Riet, B., Wampler, G.L., Elford, H.L., 1979. Synthesis of hydroxy- and amino-substituted benzohydroxamic acids: inhibition of ribonucleotide reductase and antitumor activity. J. Med. Chem. 22 (5), 589–592.

Vigouroux, C., Gharakhanian, S., Salhi, Y., Nguyen, T.H., Adda, N., Rozenbaum, W., Capeau, J., 1999. Adverse metabolic disorders during highly active antiretroviral treatments (HAART) of HIV disease. Diabetes Metab. 25, 383–392.

Zinkernagel, R.M., Hengartner, H., 1994. T-cell-mediated immunopathology versus direct cytolysis by virus: implications for HIV and AIDS. Immunol. Today 15, 262–268.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1999, p. 2046–2050 Vol. 43, No. 8
0066-4804/99/$04.00+0

Hydroxyurea Enhances the Activities of Didanosine, 9-[2-(Phosphonylmethoxy)ethyl]adenine, and 9-[2-(Phosphonylmethoxy)propyl]adenine against Drug-Susceptible and Drug-Resistant Human Immunodeficiency Virus Isolates

SARAH PALMER,* ROBERT W. SHAFER, AND THOMAS C. MERIGAN

Center for AIDS Research at Stanford, Stanford University Medical Center, Stanford, California 94305-5107

Received 30 October 1998/Returned for modification 8 March 1999/Accepted 13 May 1999

We assessed the effects of hydroxyurea (HU) at a concentration of 50 μM on the in vitro activities of 2′,3′-dideoxyinosine (ddI), 9-[2-(phosphonylmethoxy)ethyl]adenine (PMEA), and 9-[2-(phosphonylmethoxy)propyl]adenine (PMPA) against a wild-type human immunodeficiency virus (HIV) type 1 (HIV-1) laboratory isolate and a panel of five well-characterized drug-resistant HIV isolates. Fifty micromolar HU significantly increased the activities of ddI, PMEA, and PMPA against both the wild-type and the drug-resistant HIV-1 isolates. In fixed combinations, both ddI and PMEA were synergistic with HU against wild-type and drug-resistant viruses.

The experimental human immunodeficiency virus (HIV) reverse transcriptase (RT) inhibitors 9-[2-(phosphonylmethoxy)ethyl]adenine (PMEA) and 9-[2-(phosphonylmethoxy)propyl]adenine (PMPA) are acyclic phosphonate analogs of AMP (3, 26, 32). Although they already contain a single phosphate, PMEA and PMPA, like other nucleoside analogs, rely on intracellular kinases for phosphorylation to their active diphosphate forms (27, 28). The diphosphates of PMEA and PMPA and the triphosphate of 2′,3′-dideoxyinosine (ddI) (ddATP) compete with the cellular nucleotide dATP for the active binding sites on the RT enzyme (1, 8). Therefore, the antiretroviral activities of PMEA, PMPA, and ddI are dependent on two factors: (i) the activities of intracellular phosphorylating enzymes and (ii) the ratio of the amount of phosphorylated drug to the amount of competing intracellular nucleoside triphosphate pools.

The anticancer agent hydroxyurea (HU) is used for the treatment of myleoproliferative disorders (9, 34). HU is a potent inhibitor of the cellular enzyme ribonucleotide reductase, which catalyzes the reduction of ribonucleotides to deoxyribonucleotides (14). Cells exposed to HU show measurable reductions in several deoxynucleotide pools, with the reduction of dATP pools being the most pronounced (4, 10–12, 24). These decreases in deoxynucleotide pools effectively block cellular DNA synthesis (4).

HU increases the anti-HIV activities of ddI and 2′-β-fluoro-2′,3′-dideoxyadenosine, probably due to the favorable shift in the ratio of adenosine drug triphosphates versus competing cellular dATP pools which favors the binding of drug triphosphates to RT (4, 10–13, 18, 24). Due to these promising in vitro results, several clinical trials of ddI in combination with HU have been initiated (5–7, 17, 35, 36).

In the present study, we investigated the effects of HU on the anti-HIV activities of the three adenosine analogs PMEA, PMPA, and ddI. We assessed the interaction of HU with these drugs against wild-type HIV and versus a panel of drug-resistant HIV strains. We also analyzed the cytotoxicity of HU alone and in combination with PMEA, PMPA, or ddI.

HIV-1 strains. The antiviral activities of the drugs and drug combinations were assessed against six different HIV type 1 (HIV-1) strains: a wild-type laboratory isolate (HIV_{NL4-3}), three recombinant isolates containing ddI resistance mutations (HIV_{K65R}, HIV_{L74V}, and $HIV_{L74V,\ M184V}$), one molecularly constructed multinucleoside-resistant strain ($HIV_{V75I,\ F77L,F116Y,\ Q151M}$) (15), and a recently reported multidrug-resistant clinical isolate containing six major RT mutations ($HIV_{M41L,\ D67N,\ M184V,\ L210W,\ T215Y,\ K219N}$) (30).

Sequence analysis of HIV-1 strains. A 1.3-kb fragment of cDNA encompassing HIV-1 protease and the first 300 codons of RT was sequenced from each cultured supernatant as described previously (38). Briefly, purified viral RNA (Qiagen Viral RNA Extraction Kits Qiagen, Chatsworth, Calif.) was reverse transcribed and amplified by PCR with the Superscript-One-Step-RT-PCR Reagent (Life Technologies, Gaithersburg, Md.) and two primers, MAW-26 and RT21 (23). A 5-μl aliquot of the first PCR product was used for a second-round nested PCR with primers PRO-1 (29) and RT20 (23). Approximately 70 ng of the 1.3-kb product was sequenced by dye-labelled dideoxyterminator cycle sequencing (Applied Biosystems, Foster City, Calif.). Isolate sequences were compared to both patient plasma sequences and the consensus B sequence from the Los Alamos HIV Sequence Database (21).

Drug susceptibility assays. In vitro drug susceptibility assays were performed by a modified AIDS Clinical Trials Group–U.S. Department of Defense consensus method (virology manual for ACTG HIV laboratories, 1997). Peripheral blood mononuclear cells (PBMCs) were preinfected with titrated viral stocks for 4 h at 37°C in a humidified atmosphere of 5% CO_2. Each microtiter plate well contained 100,000 preinfected PBMCs and eight serial drug dilutions in cell media of ddI, PMEA, PMPA, 3′-azido-3′-deoxythymidine (AZT), 2′-deoxy-3′-thiacytidine (3TC), or indinavir (IDV) in the presence or absence of 50 μM HU. A 50 μM concentration of HU was

* Corresponding author. Present address: Southern Research Institute/Serquest, Department of Infectious Disease Research, 431 Aviation Way, Frederick, MD 21701-4756. Phone: (301) 694-3232. Fax: (301) 694-7223. E-mail: palmer@SRI.org.

TABLE 1. Effect of HU on antiviral activities of ddI, PMEA, and PMPA against a wild-type laboratory isolate and drug-resistant HIV-1 strains

Viral strain or isolate and presence of HU	IC_{50} (μM)[a] in culture		
	ddI	PMEA	PMPA
$HIV\text{-}1_{NL4\text{-}3}$			
Without HU	0.38 ± 0.06	3.1 ± 0.77	1.3 ± 0.02
With 50 μM HU	<0.05 (>8)[b]	0.37 ± 0.12 (8)	<0.05 (>26)
$HIV\text{-}1_{K65R}$[c]			
Without HU	2.3 ± 0.8	13.4 ± 1.3	4.9 ± 0.9
With 50 μM HU	<0.05 (>46)	0.10 ± 0.1 (134)	0.08 ± 0.1 (61)
$HIV\text{-}1_{L74V}$			
Without HU	1.2 ± 0.3	4.0 ± 0.2	1.7 ± 0.5
With 50 μM HU	<0.05 (>24)	0.29 ± 0.03 (14)	<0.05 (>34)
$HIV\text{-}1_{L74V, M184V}$			
Without HU	3.9 ± 0.06	1.6 ± 0.5	1.1 ± 0.1
With 50 μM HU	<0.05 (>78)	<0.05 (>32)	<0.05 (>22)
$HIV\text{-}1_{V75I, F77L, F116Y, Q151M}$			
Without HU	38 ± 3.9	8.7 ± 0.3	6.4 ± 0.58
With 50 μM HU	1.7 ± 0.45 (22)	1.9 ± 0.39 (5)	0.2 ± 0.06 (32)
$HIV\text{-}1_{M41L, D67N, M184V, L210W, T215Y, K219N}$			
Without HU	3.5 ± 0.6	5.1 ± 0.64	2.6 ± 0.72
With 50 μM HU	0.2 ± 0.15 (18)	1.4 ± 0.42 (4)	0.4 ± 0.02 (7)

[a] Results are expressed as the means ± standard deviations of four to six values obtained in two to three different experiments.
[b] Values in parentheses are the fold decrease in IC_{50} of each compound in the presence of HU. The HU-induced decrease in drug IC_{50}s were statistically significant ($P < 0.001$; two tailed t test).
[c] Subscripts are resistance mutations of the RT gene.

used since it is in the range of the average steady-state HU concentration in serum during HIV treatment (35 to 56 μM) (37). An 8:1 series of combinations of ddI and HU or PMEA and HU was also analyzed. The drug dilutions were chosen to span the 50% inhibitory concentration (IC_{50}) of each single drug (2, 3, 25, 26, 32). The drugs were combined in fixed clinically achievable ratios, based on the relative potencies of the drugs, by the median-effect method of analyzing drug interactions. Control wells containing cells and virus were coincubated on each plate.

To enable assay standardization and comparison, the 50% tissue culture infective dose of each isolate was maintained at between 30 and 100. After a 7-day incubation at 37°C and in a humidified atmosphere of 5% CO_2, viral growth was determined by a p24 antigen assay with supernatants (Dupont Pharmaceuticals, Wilmington, Del.). The percent inhibition of viral growth compared to the viral growth in the control wells without drugs was calculated. Results were expressed as the mean IC_{50} of four to six values obtained in two to three different experiments per isolate.

The results for the two-drug combinations were calculated by using a computer program that follows the median-effect principle. The computer constructs a median-effect plot of log fraction affected/fraction unaffected against the log of the dose of the two separate drugs and the dose of the combination. A combination index (CI), which compares the amount of drug which gives a 50% effect when used in combination with that which gives a 50% effect when the drug is used alone, is calculated. A CI of <1 indicates synergy, and a CI of >1 indicates antagonism. However, in accordance with variation in raw data, CIs of between 0.8 and 1.2 were considered to represent additivism.

Cytotoxicity assays. Thymidine uptake analyses were used to assess the effects of the drugs on cellular DNA synthesis. Phytohemagglutinin-stimulated PBMCs were plated at 100,000 cells per well and exposed to 3.1, 12.5, and 50 μM concentrations of ddI, PMEA, or PMPA in the presence or absence of HU at concentrations of 25 to 500 μM. Control cells, without drugs, were coincubated on each plate and were used for comparison when measuring inhibition of cellular DNA synthesis by the drugs. The plates were incubated at 37°C for 7 days. Sixteen hours prior to cell harvest, 50 μCi of [^{3}H]thymidine was introduced into all wells. The cells were harvested onto preprinted filter paper with rinsings of water and 95% ethanol. After drying at 37°C, scintillant was added and the counts on the filters were determined with a Wallac beta counter (LKB Wallac, Turku, Finland).

The presence of 50 μM HU decreased the IC_{50}s of ddI in vitro, which enhanced the anti-HIV activity of ddI against all viral strains analyzed (Table 1). The IC_{50} of ddI for many of the ddI-resistant viral strains was reduced to less than the range for the wild type in the presence of HU. These observations are consistent with previous studies showing that HU at concentrations of 50 to 100 μM increased the activity of ddI (11, 12). Moreover, recent clinical studies have shown that patients who respond to ddI and HU therapy may harbor ddI-resistant viral strains (7). Further in vitro analysis found that these resistant strains are phenotypically sensitive to inhibition by ddI and HU (19). Similar to ddI, the IC_{50}s of the acyclic adenosine derivatives PMEA and PMPA were decreased by the presence of 50 μM HU for all viruses analyzed, including five drug-resistant strains: HIV_{K65R}, HIV_{L74V}, $HIV_{L74V, M184V}$; $HIV_{V75I, F77L, F116Y, Q151M}$; and $HIV_{M41L, D67N, M184V, L210W, T215Y, K219N}$.

TABLE 2. Antiviral susceptibilities of a laboratory HIV-1 isolate and drug-resistant isolates to two-drug combinations

Isolate and drug	IC_{50} (μM)[a]		
	ddI	PMEA	HU
$HIV\text{-}1_{NL4\text{-}3}$			
Single drugs	0.38	3.1	89
ddI-HU (1:8)	0.24		2.0
PMEA-HU (1:8)		1.3	10
$HIV\text{-}1_{V75I,\ F77L,\ F116Y,\ Q151M}$[b]			
Single drugs	38	8.7	83
ddI-HU (1:8)	5.1		41
PMEA-HU (1:8)		3.0	24
$HIV\text{-}1_{M41L,\ D67N,\ M184V,\ L210W,\ T215Y,\ K219N}$			
Single drugs	3.5	5.1	76
ddI-HU (1:8)	1.2		9.7
PMEA-HU (1:8)		1.9	15

[a] All experiments were performed at least twice. The variations in the raw data were <20%.

[b] Subscripts are resistance mutations of the RT gene.

The HU-induced reductions in IC_{50}s of ddI, PMEA, and PMPA for the wild-type isolate were from 8- to >26-fold (Table 1), whereas in parallel experiments the reduction in IC_{50}s of AZT, 3TC, and IDV for the wild type were between 2- and 5-fold (data not shown). The HU-induced fold decrease in IC_{50}s of these drugs for the wild-type strain could be ranked as PMPA ≥ ddI > PMEA > AZT > 3TC > IDV. The differences of these drug IC_{50}s may be attributed to the effects of HU, a known inhibitor of ribonucleotide reductase, upon intracellular nucleotide pools (4, 10–12, 24). Cells exposed to HU experience a severe loss in dATP pools. After 5 days of continuous exposure to HU, the levels of dATP pools remain lower than those in control cells not exposed to HU (11). In contrast, studies show that natural dTTP and dCTP pools and the thymidine and deoxycytidine phosphorylating enzymes are elevated in cells exposed to HU (4, 10–12, 24). Consequently, in HU-treated cells, the ratio of phosphorylated adenosine analogs (ddATP, PMEA diphosphosphate, or PMPA diphosphate) to natural dATP may be substantially higher than the ratios of AZT-triphosphate/dTTP or 3TC-triphosphate/dCTP. The shift of the phosphorylated adenosine analogs/dATP ratio favors the binding of the analog to RT and is the probable cause of the more pronounced effect of HU on the anti-HIV activities of the adenosine analogs (ddI, PMEA, and PMPA) versus AZT and 3TC. The anti-HIV activity of IDV is independent of intracellular nucleotide levels, which may explain the limited effect of HU upon the activity of this protease inhibitor. Furthermore, HU at a concentration of 50 μM was found to inhibit viral growth by approximately 30%, or 0.3-fold; therefore, the fold decreases in drug IC_{50}s were not overly influenced by the inherent anti-HIV activity of HU.

The HU-induced decreases in drug IC_{50}s were greatest for the HIV_{K65R}, HIV_{L74V}, and $HIV_{L74V,\ M184V}$ recombinant isolates (Table 1). Recent studies have shown that the specific activity is diminished for mutant RT enzymes containing ddI-resistant mutations including enzymes with K65R or L74V mutations (20, 31). The combination of reduced specific activity and HU-induced reduction in cellular nucleotide pools may cause the increased susceptibilities of these recombinants to inhibition by antiretroviral drugs in the presence of HU.

The IC_{50}s of HU remained relatively constant (approximately 83 μM) for wild-type and resistant viral strains. These observations suggest that the RT gene mutations of the viral strains in this study have little effect on the inherent anti-HIV activity of HU (Table 2).

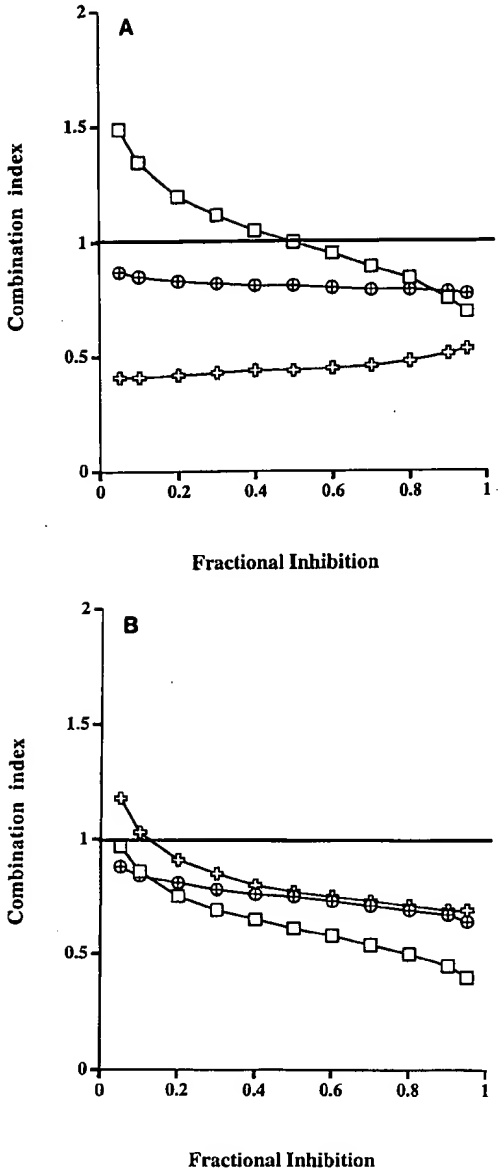

FIG. 1. Inhibition of HIV-1 isolates, a wild-type strain ($HIV_{NL4\text{-}3}$ [□]) and two drug-resistant strains ($HIV_{V75I,\ F77L,\ F116Y,\ Q151M}$ [⊕] and $HIV_{M41L,\ D67N,\ M184V,\ L210W,\ T215Y,\ K219N}$ [⊕]), by the combinations ddI-HU (A) and PMEA-HU (B). The variations in the raw data were <20%.

At clinically achievable concentrations, the combinations ddI-HU (1:8) and PMEA-HU (1:8) synergistically inhibited the two drug-resistant viral strains tested, whereas the combination of ddI-HU synergistically inhibited the wild-type isolate at high concentrations of drug (Fig. 1). The mechanism for the synergistic interactions is unknown and may reflect the different modes of action of HU (decreasing cellular nucleotides) and the nucleoside and nucleotide analogs (RT inhibition) (16, 33).

Analysis of thymidine uptake assay results revealed a reduction in cellular DNA synthesis in the presence of HU (Table

TABLE 3. Effects of ddI, PMEA, and PMPA on the thymidine uptake of PHA-stimulated PMBCs in the presence or absence HU at designated concentrations

Drug and drug concn (μM)	Counts per minute (10^4)				
	0 HU	25 μM HU	50 μM HU	100 μM HU	500 μM HU
ddI					
0	1.2 ± 0.1	1.0 ± 0.08	0.86 ± 0.07	0.68 ± 0.03	0.04 ± 0.006
3.1	1.3 ± 0.07	0.9 ± 0.1	0.9 ± 0.03	0.5 ± 0.04	0.03 ± 0.006
12.5	1.1 ± 0.1	0.9 ± 0.08	0.9 ± 0.1	0.5 ± 0.1	0.03 ± 0.007
50	0.9 ± 0.1	0.8 ± 0.009	0.8 ± 0.09	0.5 ± 0.05	0.03 ± 0.006
PMEA					
0					
3.1	1.0 ± 0.1	1.0 ± 0.06	0.6 ± 0.06	0.4 ± 0.02	0.04 ± 0.006
12.5	0.9 ± 0.09	0.8 ± 0.03	0.6 ± 0.04	0.4 ± 0.04	0.03 ± 0.003
50	0.5 ± 0.03	0.5 ± 0.06	0.5 ± 0.04	0.2 ± 0.01	0.03 ± 0.005
PMPA					
0					
3.1	1.2 ± 0.2	1.0 ± 0.05	0.8 ± 0.02	0.5 ± 0.06	0.04 ± 0.003
12.5	1.2 ± 0.2	0.9 ± 0.06	0.8 ± 0.1	0.5 ± 0.1	0.04 ± 0.003
50	1.1 ± 0.1	0.9 ± 0.1	0.8 ± 0.02	0.5 ± 0.03	0.04 ± 0.005

3). Although the measurements of thymidine uptake may be affected by the HU-induced increase in intracellular dTTP pools and the extended half-life of these pools in the presence of HU, this observation suggests that HU has a cytostatic effect on cells (4, 22).

In conclusion, the presence of HU at low, clinically tolerated concentrations enhances the anti-HIV activities of ddI, PMEA, and PMPA. This HU-induced increase in the activities of ddI, PMEA, and PMPA against HIV is also observed in clinical isolates that are resistant to one or more of these compounds. The two-drug combinations ddI-HU and PMEA-HU synergistically inhibited drug-resistant viral strains. This study provides evidence that supports the need for clinical trials with HU (i) in combination with ddI against ddI-resistant patient isolates and (ii) in combination with two recently developed adenosine analogs, PMEA and PMPA. Moreover, the strategy of combining highly specific HIV inhibitors with relatively nonspecific inhibitors is an approach to drug resistance that should be tested clinically.

We thank Gilead Sciences, Foster City, Calif., for the kind gifts of PMEA and PMPA. We especially thank Darcy Levee and Kristi Cooley for excellent technical assistance.

REFERENCES

1. **Arts, E. J., and M. A. Wainberg.** 1994. Preferential incorporation of nucleoside analogs after template switching during human immunodeficiency virus reverse transcription. Antimicrob. Agents Chemother. **38**:1008–1016.
2. **Balzarini, J., L. Naesens, J. Slachmuylders, H. Niphuis, I. Rosenberg, A. Holy, H. Schellekens, and E. De Clercq.** 1991. 9-(2-Phosphonylmethoxyethyl)adenine (PMEA) effectively inhibits retrovirus replication *in vitro* and simian immunodeficiency virus infection in rhesus monkeys. AIDS **5**:21–28.
3. **Balzarini, J., T. Vahlenkamp, H. Egberink, K. Hartmann, M. Witvrouw, C. Pannecouque, P. Casara, J. F. Nave, and E. De Clercq.** 1997. Antiretroviral activities of acyclic nucleoside phosphonates [9-(2-phosphonylmethoxyethyl)adenine, 9-(2-phosphonylmethoxyethyl)guanine, (R)-9-(2-phosphonylmethoxypropyl)adenine, and MDL 74,968] in cell cultures and murine sarcoma virus-infected newborn NMRI mice. Antimicrob. Agents Chemother. **41**:611–616.
4. **Bianchi, V., E. Pontis, and P. Reichard.** 1986. Changes of deoxyribonucleoside triphosphate pools induced by hydroxyurea and their relation to DNA synthesis. J. Biol. Chem. **261**:16037–16042.
5. **Biron, F., F. Lucht, D. Peyramond, A. Fresard, T. Vallet, F. Nugier, J. Grange, S. Malley, F. Hamedi-Sangsari, and J. Vila.** 1995. Anti-HIV activity of the combination of didanosine and hydroxyurea in HIV-1-infected individuals. J. Acquired Immune Defic. Syndr. Hum. Retrovirol. **10**:36–40.
6. **Biron, F., F. Lucht, D. Peyramond, A. Fresard, T. Vallet, F. Nugier, J. Grange, S. Malley, F. Hamedi-Sangsari, and J. Vila.** 1996. Pilot clinical trial of the combination of hydroxyurea and didanosine in HIV-1 infected individuals. Antivir. Res. **29**:111–113.
7. **De Antoni, A., A. Foli, J. Lisziewicz, and F. Lori.** 1997. Mutations in the pol gene of human immunodeficiency virus type 1 in infected patients receiving didanosine and hydroxyurea combination therapy. J. Infect. Dis. **176**:899–903.
8. **De Clercq, E.** 1992. HIV inhibitors targeted at the reverse transcriptase. AIDS Res. Hum. Retroviruses **8**:119–134.
9. **Donehower, R. C.** 1992. An overview of the clinical experience with hydroxyurea. Semin. Oncol. **19**(Suppl. 9):11–19.
10. **Gao, W.-Y., A. Cara, R. C. Gallo, and F. Lori.** 1993. Low levels of deoxynucleotides in peripheral blood lymphocytes: a strategy to inhibit human immunodeficiency virus type 1 replication. Proc. Natl. Acad. Sci. USA **90**: 8925–8928.
11. **Gao, W.-Y., D. G. Johns, S. Chokekijchai, and H. Mitsuya.** 1995. Disparate actions of hydroxyurea in potentiation of purine and pyrimidine 2′,3′-dideoxynucleoside activities against replication of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **92**:8333–8337.
12. **Gao, W.-Y., D. G. Johns, and H. Mitsuya.** 1994. Anti-human immunodeficiency virus type 1 activity of hydroxyurea in combination with 2′,3′-dideoxynucleosides. Mol. Pharmacol. **46**:767–772.
13. **Gao, W. Y., H. Mitsuya, J. S. Driscoll, and D. G. Johns.** 1995. Enhancement by hydroxyurea of the anti-human immunodeficiency virus type 1 potency of 2′-beta-fluoro-2′,3′-dideoxyadenosine in peripheral blood mononuclear cells. Biochem. Pharmacol. **50**:274–276.
14. **Hurta, R. A., and J. A. Wright.** 1990. Amplification of the genes for both components of ribonucleotide reductase in hydroxyurea resistant mammalian cells. Biochem. Biophys. Res. Commun. **167**:258.
15. **Iversen, A. K. N., R. W. Shafer, K. Wehrly, M. A. Winters, J. I. Mullins, B. Chesebro, and T. C. Merigan.** 1996. Multidrug-resistant human immunodeficiency virus type 1 strains resulting from combination antiretroviral therapy. J. Virol. **70**:1086–1090.
16. **Johns, D. G., and W. Y. Gao.** 1998. Selective depletion of DNA precursors: an evolving strategy for potentiation of dideoxynucleoside activity against human immunodeficiency virus. Biochem. Pharmacol. **55**:1551–1556.
17. **Lori, F., H. Jessen, A. Foli, J. Lisziewicz, and P. S. Matteo.** 1997. Long-term suppression of HIV-1 by hydroxyurea and didanosine. JAMA **277**:1437–1438. (Letter.)
18. **Lori, F., A. Malykh, A. Cara, D. Sun, J. N. Weinstein, J. Lisziewicz, and R. C. Gallo.** 1994. Hydroxyurea as an inhibitor of human immunodeficiency virus-type 1 replication. Science **266**:801–805.
19. **Lori, F., A. G. Malykh, A. Foli, R. Maserati, A. De Antoni, L. Minoli, D. Padrini, A. Degli Antoni, E. Barchi, H. Jessen, M. A. Wainberg, R. C. Gallo, and J. Lisziewicz.** 1997. Combination of a drug targeting the cell with a drug targeting the virus controls human immunodeficiency virus type 1 resistance. AIDS Res. Hum. Retroviruses **13**:1403–1409.
20. **Miller, M. D., P. D. Lamy, M. D. Fuller, A. S. Mulato, N. A. Margot, T. Cihlar, and J. M. Cherrington.** 1998. Human immunodeficiency virus type 1 reverse transcriptase expressing the K70E mutation exhibits a decrease in specific activity and processivity. Mol. Pharmacol. **54**:291–297.

21. **Myers, G., B. Korber, B. Foley, K.-T. Jeang, J. W. Mellors, and S. Wain-Hobson.** 1996. Human retroviruses and AIDS 1996: a compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, N.M.
22. **Nicander, B., and P. Reichard.** 1985. Relations between synthesis of deoxyribonucleotides and DNA replication in 3T6 fibroblasts. J. Biol. Chem. **260**:5376–5381.
23. **Nijhuis, M., C. A. B. Boucher, and R. Schuurman.** 1995. Sensitive procedure for amplification of HIV-1 RNA using a combined reverse transcriptase and amplification reaction. BioTechniques **19**:178–182.
24. **Palmer, S., and S. Cox.** 1997. Increased activation of the combination of 3′-azido-3′-deoxythymidine and 2′-deoxy-3′-thiacytidine in the presence of hydroxyurea. Antimicrob. Agents Chemother. **41**:460–464.
25. **Palmer, S., J. Harmenberg, and S. Cox.** 1996. Synergistic inhibition of HIV isolates (including 3′-azido-3′-deoxythymidine-resistant isolates) by foscarnet in combination with 2′,3′-dideoxyinosine or 2′,3′-dideoxycytidine. Antimicrob. Agents Chemother. **40**:1285–1288.
26. **Pauwels, R., J. Balzarini, D. Schols, M. Baba, J. Desmyter, I. Rosenberg, A. Holy, and E. De Clercq.** 1988. Phosphonylmethoxyethyl purine derivatives, a new class of anti-human immunodeficiency virus agents. Antimicrob. Agents Chemother. **32**:1025–1030.
27. **Robbins, B. L., J. Greenhaw, M. C. Connelly, and A. Fridland.** 1995. Metabolic pathways for activation of the antiviral agent 9-(2-phosphonylmethoxyethyl)adenine in human lymphoid cells. Antimicrob. Agents Chemother. **39**:2304–2308.
28. **Robbins, B. L., R. V. Srinivas, C. Kim, N. Bischofberger, and A. Fridland.** 1998. Anti-human immunodeficiency virus activity and cellular metabolism of a potential prodrug of the acyclic nucleoside phosphonate 9-*R*-(2-phosphonomethoxypropyl)adenine (PMPA), bis(isopropyloxymethylcarbonyl) PMPA. Antimicrob. Agents Chemother. **42**:612–617.
29. **Schapiro, J. M., M. A. Winters, F. Stewart, B. Efron, J. Norris, M. J. Kozal, and T. C. Merigan.** 1996. The effect of high-dose saquinavir on viral load and CD4 T-cell counts in HIV-infected patients. Ann. Intern. Med. **124**:1039–1050.
30. **Shafer, R. W., M. A. Winters, S. Palmer, and T. C. Merigan.** 1998. Multiple concurrent reverse transcriptase and protease mutations and multidrug resistance of HIV-1 isolates from heavily treated HIV-1 infected patients. Ann. Intern. Med. **128**:906–911.
31. **Sharma, P. L., and C. S. Crumpacker.** 1997. Attenuated replication of human immunodeficiency virus type 1 with a didanosine-selected reverse transcriptase mutation. J. Virol. **71**:8846–8851.
32. **Smith, M. S., E. L. Brian, E. De Clercq, and J. S. Pagano.** 1989. Susceptibility of human immunodeficiency virus type 1 replication in vitro to acyclic adenosine analogs and synergy of the analogs with 3′-azido-3′-deoxythymidine. Antimicrob. Agents Chemother. **33**:1482–1486.
33. **Sommadossi, J. P.** 1993. Nucleoside analogs: similarities and differences. Clin. Infect. Dis. **16**(Suppl. 1):S7–S15.
34. **Tracewell, W. G., D. L. Trump, W. P. Vaughan, D. C. Smith, and P. R. Gwilt.** 1995. Population pharmacokinetics of hydroxyurea in cancer patients. Cancer Chemother. Pharmacol. **35**:417–422.
35. **Vila, J., F. Biron, F. Nugier, T. Vallet, and D. Peyramond.** 1996. 1-year follow-up of the use of hydroxycarbamide and didanosine in HIV infection. Lancet **348**:203–204. (Letter.)
36. **Vila, J., F. Nugier, G. Bargues, T. Vallet, D. Peyramond, F. Hamedi-Sangsari, and J. M. Seigneurin.** 1997. Absence of viral rebound after treatment of HIV-infected patients with didanosine and hydroxycarbamide. Lancet **350**:635–636. (Letter.)
37. **Villani, P., R. Maserati, M. B. Regazzi, R. Giacchino, and F. Lori.** 1996. Pharmacokinetics of hydroxyurea in patients infected with human immunodeficiency virus type I. J. Clin. Pharmacol. **36**:117–121.
38. **Winters, M. A., J. M. Schapiro, J. Lawrence, and T. C. Merigan.** 1998. HIV-1 protease genotypes and in vitro protease inhibitor susceptibility from long-term saquinavir-treated individuals who switched to other protease inhibitors. J. Virol. **72**:5303–5306.